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THE PURIFICATION AND CHARACTERIZATION OF THREONINE DEHYDROGENASE FROM *KLEBSIELLA PNEUMONIAE* AND *ENTEROBACTER AEROGENES*

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ABSTRACT

Threonine dehydrogenase has been purified to apparent homogeneity from *Klebsiella pneumoniae* and *Enterobacter aerogenes*. Purification was achieved by sequential chromatography on DEAE ion exchange, Blue Sepharose affinity gel, and Toyopearl HW-55s gel filtration. The amino-terminus for the enzyme in both organisms has been sequenced and placed the enzyme from *K. pneumoniae* with a class of long chain alcohol dehydrogenase family. The subunit molecular mass was estimated as 38.8 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, while an apparent native molecular mass of 154 kDa was shown by gel filtration chromatography, suggesting a tetrameric structure. As for the enzyme from *E. aerogenes*, it is described as a possible dimer with a native molecular mass estimate of 104.5 kDa, and a subunit molecular mass estimate of 43.1 kDa. This enzyme showed homology to a group of alcohol dehydrogenase I family with a preference to propanol as a substrate.

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I am eternally grateful to my family who never stopped encouraging and supporting me to accomplish my goals and very often extended their arms in many ways to provide me with the help that I certainly needed.

Thank you...

بسم الله الرحمن الرحيم

الى أمي وأبي مع خالص حبي واحترامي

الى من أحبها قلبي فسعى إليها
والى من رأتها عيناي فجريا إليها
الى شريكة عمري الذي إن دام لم يكفي
أن أريها شيئاً قليلاً من عشقي إليها

أهدي هذه الأطروحة لأعبر بشيء قليل عن حبي إليكم جميعاً

ABBREVIATIONS

- ADH — Alcohol dehydrogenase
- AKB — 2-Amino-3-ketobutyrate
- AKB Ligase — 2-Amino-3-ketobutyrate:CoASH Ligase
- AP — Alkaline phosphatase
- BCIP — 5-Bromo-4-chloro-3-indolyl phosphate
- BLAST — Basic Local Alignment Search Tool
- BSA — Bovine serum albumin
- DEAE — Diethylaminoethyl
- DNA — Deoxyribonucleic acid
- dNTP — 2'-Deoxy-nucleoside-5'-triphosphate
- *Ea* — *Enterobacter aerogenes*
- EDTA — Ethylenediaminetetra-acetate
- g — gram
- GCG — Genetic Computer Group
- kb — Kilobase
- kDa — kilodalton
- *Kp* — *Klebsiella pneumoniae*
- L — Liter
- LB — Luria Broth
- Min — Minute
- MW — Molecular weight

- N-terminal — Amino-terminal
- NAD^+ — Nicotinamide-adenine dinucleotide (oxidized form)
- NADH — Nicotinamide-adenine dinucleotide (reduced form)
- NBT — Nitro blue tetrazolium
- NCBI — National Center for Biotechnology Information
- PCR — Polymerase chain reaction
- PhMS — Phenazene mthosulfate
- pI — Isoelectric point
- PITC — Phenylisothiocyanate
- pITV — para-iodonitrotetrazolium violet
- PTC — Phenylthiocarbamyl
- PTH — 3-phenyl-2-thiohydantion
- R_m — Relative migration
- RNA — Ribonucleic acid
- RNAase — Ribonuclease
- rpm — Revolution per minute
- SA-AP — Streptavidin-alkaline phosphatase
- SDS-PAGE — Sodium dodecyl sulfate-polyarylamide gel electrophoresis
- SSC — Sodium chloride/sodium citrate buffer
- TDH — L-Threonine dehydrogenase
- TE — Tris-EDTA buffer
- TEMED — N, N, N', N'-Tetramethylenediamine
- Tris — 2-amino-2-hydroxymethyl propane-1, 3-diol

AMINO ACID SYMBOLS AND ABBREVIATIONS

Amino acid	Three-letter abbreviation	One-letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Try	Y
Valine	Val	V

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INTRODUCTION

1. Threonine Dehydrogenase

Threonine is one of nine essential amino acids that the body can only acquire from protein-rich foods. Only plants and prokaryotes can synthesize threonine from homoserine, which is originally derived from aspartate. Homoserine undergoes phosphorylation, followed by a pyridoxal phosphate-dependent reaction that simultaneously cleaves out the phosphate and causes the hydroxyl group to migrate from the γ- to the β-carbon, see figure 1.1:

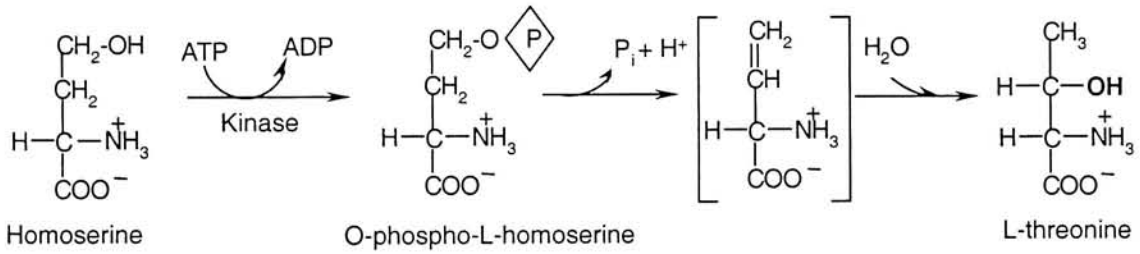


Figure 1.1
Threonine Synthesis

Threonine is degraded in three ways in the cell. Two of those pathways are initiated by threonine aldolase (figure 1.2A), and threonine dehydratase (figure 1.2B). The third pathway is initiated by threonine dehydrogenase (TDH), which is thought to play the major role in initiating threonine metabolism in both prokaryotes[1,2] and eukaryotes.[3]

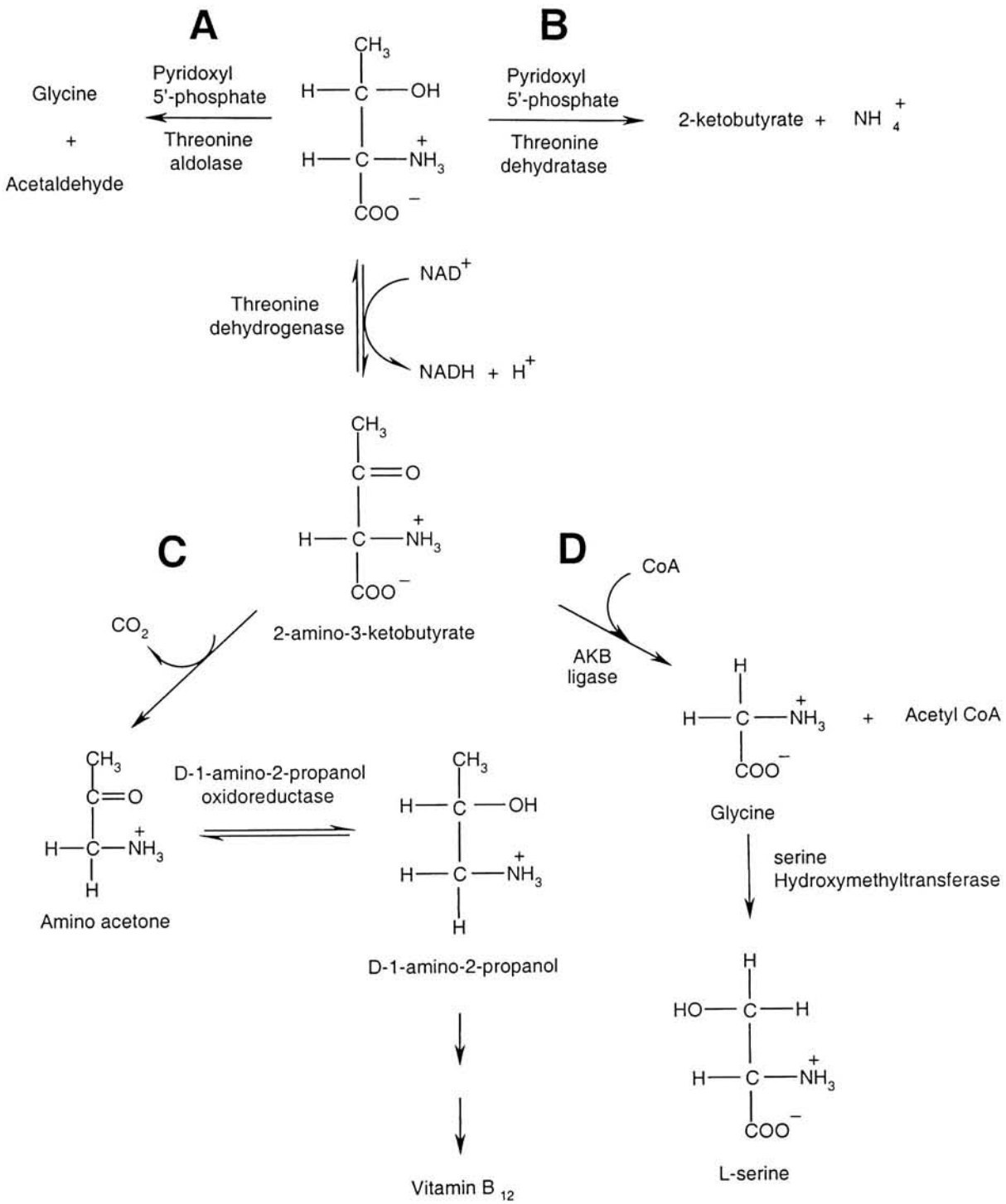


Figure 1.2
Threonine degradation pathways

It is the only pathway for L-threonine degradation that is detected in the chicken liver.[4] In addition, it accounts for 87% of the L-threonine degraded in liver of normally fed rats.[5] TDH is an enzyme that catalyzes the oxidation of L-threonine to the unstable intermediate 2-amino-3-ketobutyrate (AKB), which in turn either

(a) spontaneously decarboxylates to form aminoacetone, which can be sterospecifically reduced to D-1-amino-2-propanol and incorporated in vitamin B_{12} synthesis [6] (figure. 1.2C);

(b) is converted to acetyl CoA plus glycine by the enzyme aminoketobutyrate ligase (AKB ligase).[7] In some organisms, glycine is subsequently converted to serine in a reaction catalyzed by serine hydroxymethyltransferase (figure. 1.2D);

TDH is a member of the alcohol dehydrogenase (ADH) family. Alcohol dehydrogenase (EC 1.1.1.1), the prototype enzyme from this class, catalyzes the reversible oxidation of ethanol to acetaldehyde with the concomitant reduction of NAD [8] Currently, three structurally and catalytically different types of alcohol dehydrogenases are known:

Zinc-containing 'long-chain' alcohol dehydrogenases.

Insect-type, or 'short-chain' alcohol dehydrogenases.

Iron-containing alcohol dehydrogenases.

Zinc-containing ADHs [9,10] are dimeric or tetrameric enzymes that bind two atoms of zinc per subunit. One of the zinc atoms is essential for catalytic activity while the

other has a structural purpose. Both zinc atoms are coordinated by either cysteine or histidine residues; the catalytic zinc is coordinated by two cysteines and one histidine.

Zinc-containing ADHs are found in bacteria, mammals, plants, and in fungi. *Escherichia coli*'s TDH has been shown to be a member of the zinc containing long-chain alcohol dehydrogenases.[11, 12] The enzyme has also been sequenced from other organisms and shown to have similar characteristics to TDH from *E. coli*. Some of those bacterial organisms are: *Xanthomonas campestris*,[13] *Deinococcus radiodurans*,[14] *Streptomyces coelicolor*,[15] *Rhizobium meliloti*,[16] *Bacillus subtilis*[17], and *Pyrococcus abyssi*. Since *E. coli* is one of the widely researched organism and its TDH remains the most thoroughly investigated, a TDH sequence homology comparison between *E. coli* and the rest of the organisms was generated using the Basic Local Alignment Search Tool (BLAST)[18, 19] as shown in table 1.1:

Table 1.1
TDH Sequence Comparison of *E. coli* with other organisms

Organism	Identical amino acid	Similar amino acid
Deinococcus radiodurans (Dr)	246/342 (71%)	285/342 (82%)
Rhizobium meliloti (Rm)	231/342 (67%)	270/342 (78%)
Xanthomonas campestris (Xc)	209/341 (61%)	258/341 (75%)
Streptomyces coelicolor (Sc)	196/340 (57%)	243/340 (70%)
Bacillus subtilis (Bs)	156/341 (45%)	219/341 (63%)
Pyrococcus abyssi (Pa)	138/340 (40%)	210/340 (61%)

A TDH amino acid sequence alignment (Figure 1.3) of the above organisms was constructed using the Wisconsin Package of the Genetics Computer Group (GCG). A careful examination of the aligned sequences generates several interesting findings. There are 84 strictly conserved residues and 62 highly conserved residues within this group. In addition, 53 other residues have six out of the seven amino acids either conserved or highly conserved. The seven underlined residues that are thought to be the ligands to the catalytic and structural zinc atoms are also conserved. This confirms the findings by Vallee and Auld [20] who compiled 23 different sequences of alcohol dehydrogenases and found the first cysteine of the zinc active site and the region surrounding it also homologous.

```
1                                                                  50
Ec  ~~~~~~~~~~ ~~~~~~~~~~ ~~MKALSKLK AEEGIWMTDV PVPELGHNDL
Dr  MTSPSSTATS SPTTTTAPTP ARMRALSKQQ PGEGIWMIET EVPTPGPNDL
Rm  ~~~~~~~~~~ ~~~~~~~~MT NMMKALVKTK PEVGLWMERV PVPEIGPNDV
Xc  ~~~~~~~~~~ ~~~~~~~~~~ ~~MKALVKRE ASKGIWLEQV PVPTPGPNEV
Sc  ~~~~~~~~~~ ~~~~~~~~~~ ~~MKALVKEN AEPGLWLADV PEPTIGSGDV
Bs  ~~~~~~~~~~ ~~~~~~~~MQS GKMKALMKKD GAFGAVLTEV PIPEIDKHEV
Pa  ~~~~~~~~~~ ~~~~~~~~MS EKMVAIMKTK PAYGAELVEV DVPKPGPGEV

        51                                                        100
Ec  LIKIRKTAIC GTDVHIYNWD EWSQKTIPVP MVVGHEYVGE VVGIGQEVKG
Dr  LIRIRKGSIC GTDVHIYKWD DWASQTVPVP MVVGHEYVGV VAGMGSEVRG
Rm  LIRVRKSAIC GTDVHIWNWD QWAEKTIPVP MVVGHEFMGE VVEVGPAVSK
Xc  LIKLEKTAIC GTDLHIYLWD EWSQRTITPG LTIGHEFVGR VAELGSAVTG
Sc  LIKVLRTGIC GTDLHIRAWD GWAQQAIRTP LVVGHEFVGE VVDTGRDVTD
Bs  LIKVKAASIC GTDVHIYNWD QWARQRIKTP YVFGHEFSGI VEGVGENVSS
Pa  LIKVIATSIC GTDLHIYEWN EWAQSRIKPP QIMGHEVAGE VVEVGPGVEG

        101                                                       150
Ec  FKIGDRVSGE GHITCGHCRN CRGGRTHLCR NTIGVGVNRP GCFAEYLVIP
Dr  FEIGDRVSGE GHVTCGHCRN CRAGRRHLCR NTQGVGVNRP GSFAEYLVLP
Rm  HHVGERVSGE GHIVCGKCRN CRAGRGHLCR NTLGVGVNRP GSFAEFVCLP
Xc  YQVGQRVSAE GHIVCGHCRN CRGGRPHLCP NTVGIGVNVN GAFAEYMVMP
Sc  IKAGDRVSGE GHLVCGKCRN CLAGRRHLCR ATVGLGVGRD GAFAEYVALP
Bs  VKVGEYVSAE THIVCGECVP CLTGKSHVCT NTAIIGVDTA GCFAEYVKVP
Pa  IEVGDYVSVE THIVCGKCYA CRRGQYHVCQ NTKIFGVDTD GVFAEYAVVP

        151                                                       200
Ec  AFNAFKIPDN ISDDLAAIFD PFGNAVHTAL SFDLVGEDVL VSGAGPIGIM
Dr  AFNAFKLPDD IPDDVAAIFD PFGNAVHTAL SFDLVGEDVL ITGAGPIGCM
Rm  EYNVVSIPDD VPDEIAAIFD PFGNAVHTAL SFDLVGEDVL VTGAGPIGIM
Xc  ASNLWPIPDQ IPSELAAFFD PYGNAAHCAL EFDVIGEDVL ITGAGPIGII
Sc  ASNVWVHRVP VDLDVAAIFD PFGNAVHTAL SFPLVGEDVL ITGAGPIGLM
Bs  ADNIWRNPAD MDPSIASIQE PLGNAVHTVL ESQPAGGTTA VIGCGPIGLM
Pa  AQNVWKNPKS IPPEYATLQE PLGNAVDTVL AGPISGKSVL ITGAGPLGLL

        201                                                       250
Ec  AAAVAKHVGA RNVVITDVNE YRLELARKMG ITRAVNVAKE NLNDVMA.EL
Dr  AAAVARHVGA RNVVITDVND YRLDLARQMG VTRAVNVARE DLWTVATQEL
Rm  GAMVAKRCGA RKVVITDINP VRLDLARKLG IDHVVDASKE KLADVM.RVI
Xc  AAGICKHIGA RNVVVTDVND FRLKLAADLG ATRVVNVSKT SLKDVMA.DL
Sc  AAAVARHAGA RNVVITDVSE ERLELARKVG ATLALNVSDA TIAD.GQREL
Bs  AVAVAKAAGA SQVIAIDKNE YRLRLAKQMG ATCTVSIEKE DPLKIVS.AL
Pa  GIAVAKASGA YPVIVSEPSD FRRELAKKVG ADYVINPFEE DVVKEVM.DI

        251                                                       300
Ec  GMTEGFDVGL EMSGAPPAFR TMLDTMNHGG RIAMLGIPPS DMSIDW.TKV
Dr  DMHEGFDVGM EMSGSGPAFA QMVSVMNNGG KVALLGIPSG EVQIDW.NAV
Rm  GMTEGFDVGL EMSGAAPAFR DMIDKMNNGG KIAILGIAPA GFEIDW.NKV
Xc  HM.EGFDVGL EMSGNPRAFN DMLDCMYHGG KIAMLGIMPR GAGCDW.DKI
Sc  GLREGFDIGL EMSGRPEAMR DMIANMTHGG RIAMLGLPAE EFPVDW.ARV
Bs  TSGEGADLVC EMSGHPSAIA QGLAMAANGG RFHILSLPEH PVTIDLTNKV
Pa  TDGNGVDVFL EFSGAPKALE QGLQAVTPAG RVSLLGLYPG KVSIDFNNLI

        301                                                       350
Ec  IFKGLFIKGI YGREMFETWY KMAALIQSG. LDLSPIITHR F.SIDDFQKG
Dr  IFKMLTIKGI YGREMFETWY KMAALIQSG. LDLTPVITHH Y.GIGDFQQG
Rm  IFKMLNLKGI YGREMFETWY KMIAFVQGG. LDLSPIITHR I.GIDDFRDG
Xc  IFKGLTVQGI YGRKMYETWY KMTQLVLSG. FPLQKVLTHQ L.SIDEFQKG
Sc  VTSMITVKGI YGREMFETWY AMSVLLEGG. LDLAPVITGR Y.SHRDFEAA
Bs  VFKGLTIQGI TGRKMFSTWR QVSQLISSNM IDLAPVITHQ FP.LEEFEKG
Pa  IFKALTVYGI TGRHLWETWY TVSRLLQSGK LNLDPIITHK YKGFDKYEEA
```

```
    351                     373
Ec  FDAMRSGQSG KVILSWD~~~ ~~~
Dr  FDAMLSGQSG KVILDWETEE QSA
Rm  FEAMRSGNSG KVVMDW~~~~ ~~~
Xc  FDLMEEGKAG KVVLSWN~~~ ~~~
Sc  FADAASGRGG KVILDWTA~~ ~~~
Bs  FELMRSGQCG KVILIP~~~~ ~~~
Pa  FELMRAGKTG KVVFMLK~~~ ~~~
```

Figure 1.3: Alignment[21] of amino acid sequences of known threonine dehydrogenases including *Echerichia coli* (Ec), *Deinococcus radiodurans* (Dr), *Rhizobium meliloti* (Rm), *Xanthomonas campestris* (Xc), *Streptomyces coelicolor* (Sc), *Bacillus subtilis* (Bs), Pyrococcus abyess (Pa). The 84 strictly conserved residues and the 62 highly conserved residues are indicated with blue and red colors respectively. The 53 amino acid residues (green) have six out of the seven amino acids either conserved or highly conserved. The seven residues that are involved in the enzyme catalysis and structure are underlined. The dashed lines indicate absence of amino acids either due to unknown sequences or differences in peptide length. The gaps represented by (.) were introduced to optimize alignment between sequences.

Using Distances and GrowTree programs within the Wisconsin Package software, a phylogenetic tree was generated for the above TDH sequences (figure 1.4). Distances writes a matrix of the pairwise evolutionary distances within a group of aligned sequences, while GrowTree creates a phylogenetic tree from a distance matrix created by distances using neighbor-joining method.[22]

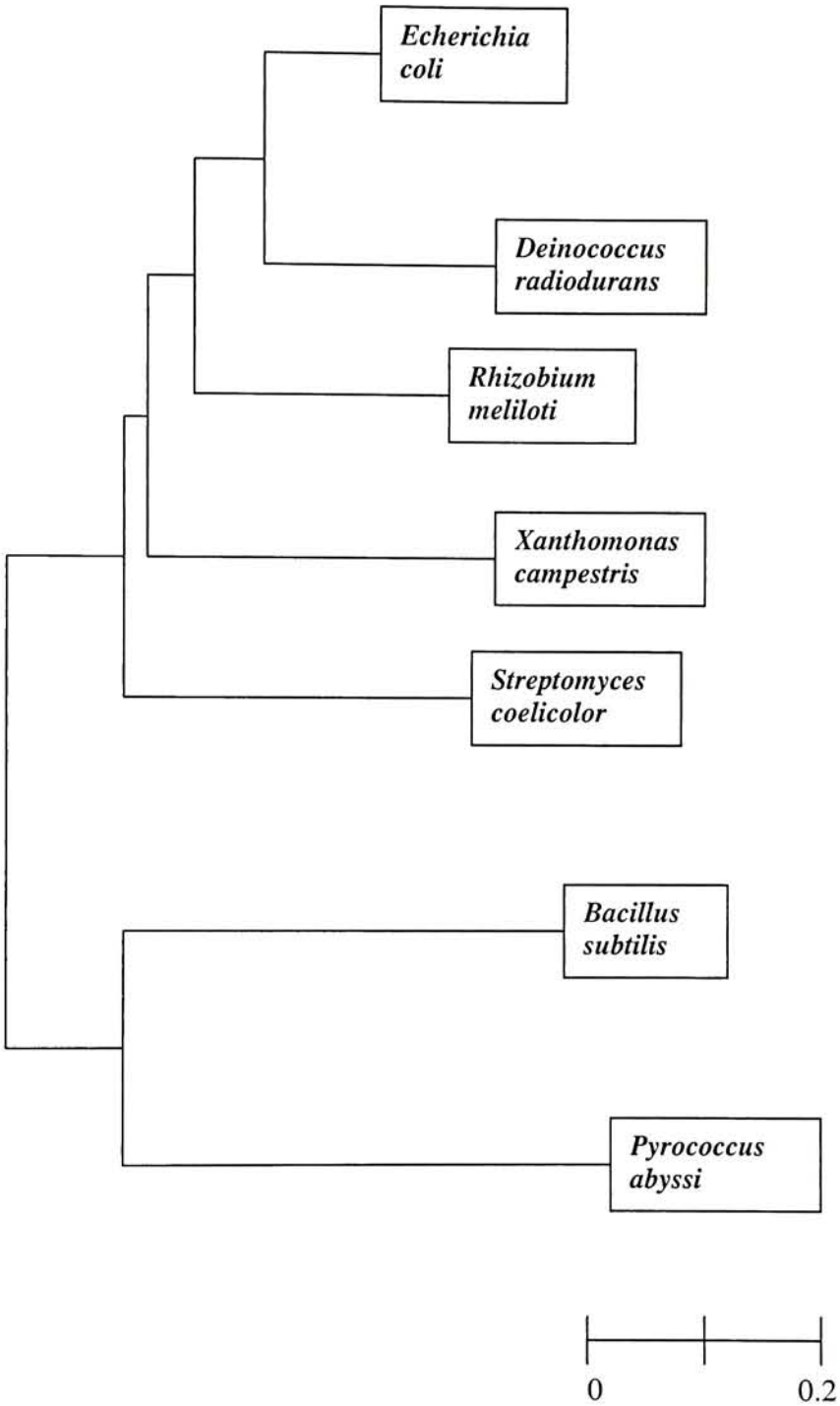


Figure 1.4
Neighbor-Joining tree for bacterial threonine dehydrogenase sequences taken from gene bank.

The main goal of this research project is the determination of threonine dehydrogenase sequence from *Klebsiella pneumoniae* and *Enterobacter aerogenes*. *K. pneumoniae* was discovered in 1882 by Friedlander.[23] It is a gram-negative rodlike nonmotile mostly lactose-fermenting bacterium found in respiratory, intestinal, and urogenital tracts of animals and men. It is associated with pneumonia and other respiratory infections, and may cause severe infections[24] such as bacteraemia and septicaemia with mortality rates ranging from 25 to 50%. Unlike other enterobacterial close relatives, such as *E .coli* and *Salmonella spp*, *K. pneumoniae* is a classical example of a species that constitutively expresses capsular polysaccharide (capsule).

Enterobacter aerogenes, on the other hand, is similar to *Klebsiella*, the key difference being that *Enterobacter* can grow on ornithine media while *K pneumoniae* cannot. Although this bacterium is part of the normal flora of the human intestinal tract, several species cause opportunistic infections of the urinary tract as well as other parts of the body. *E. aerogenes* is a pathogen that does not cause diarrhea; however, it has been associated with urinary tract and respiratory tract infections.

2. Protein Purification

In order to characterize and fully understand its physical and biological properties, a protein must be purified from its natural source. Protein purification is usually performed in a series of steps using different techniques for each step. Some techniques are more useful when handling large amounts of material, whereas others work best on small amounts. A purification procedure is arranged so that the techniques that work best with large amounts are used early in the overall purification. Hydrophobic interaction and ion-exchange are two of the techniques that are useful to start protein purification from a crude cell extract. Affinity and size exclusion techniques are commonly used toward the end of the purification protocol. The suitability of each purification step is evaluated in terms of the amount of purification achieved by that step and the percent recovery of the desired protein. In this research project, three purification methods were used in combination to achieve the desired results. These techniques are based on ion-exchange chromatography, affinity chromatography, and size exclusion chromatography.

The basis of ion-exchange chromatography is that charged ions can freely exchange with ions of the same type. In this context, the mass of the ion is irrelevant. Therefore, it is possible for a bulky anion like a negatively charged protein to exchange with chloride ions (figure 1.5). This process can later be reversed by washing with chloride ions in the form of NaCl or KCl solution. Such washing removes weakly bound proteins first, followed by more strongly bound proteins with greater net negative charge. Like most column chromatography techniques, ion-exchange chromatography requires a stationary phase, which is composed of insoluble, hydrated polymers, such as cellulose, dextran, and sephadex.[25]

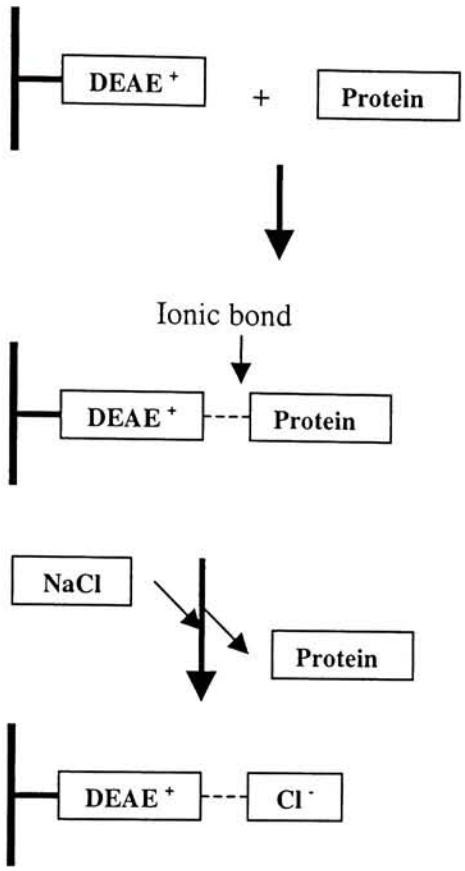


Figure 1.5 Ion-exchange chromatography of anionic protein

Affinity chromatography depends on the ability of the biological molecules to recognize complementary substances within the column. The technique may be described as an exploitation of a protein's affinities in its purification rather than its properties as a protein, such as charge, size, or hydrophobicity. The overall process of affinity chromatography may be split into a number of stages. The first stage is choosing a matrix, which is an insoluble gel. Then, the gel is activated for ligand attachment. The ligand, a molecule that will interact with the desired protein, can then be coupled to the

insoluble matrix either directly as in the case of protein ligands or via a spacer arm for small molecular weight ligands to form the affinity sorbent. Once the column is ready, the crude sample containing the desired protein is applied to the affinity column. The protein is contacted with the affinity matrix under conditions that allow the protein to bind to the ligand. The contaminating proteins can then be washed away before the conditions are changed (pH, added cofactor, added substrate) so that the protein no longer binds to the ligand and is eluted from the column.

Finally, size-exclusion chromatography (also known as gel-filtration chromatography) is a technique for separating proteins and other biological macromolecules based on their molecular size. The solid-phase matrix consists of porous beads (typically 100-250 μm) that are packed into a column with a mobile-liquid phase flowing through the column. The mobile phase has access to both the volume inside pores and outside the beads. Separation can occur when large molecules remain in the volume external to the beads because they are unable to enter the pores. The resulting shorter flow path means that they pass through the column relatively rapidly, emerging early, whereas small molecules that can access the liquid within the pores of the beads are retained longer and therefore pass more slowly through the column. Size exclusion tends to be used at the end of a purification scheme when impurities are low in number and the target protein has been purified and concentrated by earlier chromatography steps.

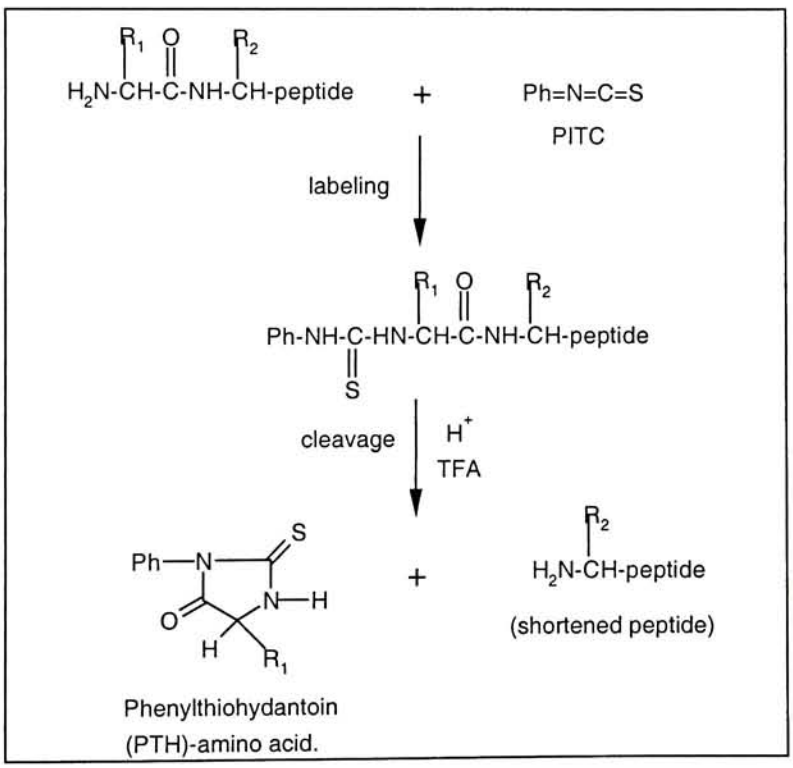
3. Protein Sequencing

It has been half a century since the Swedish chemist Pehr Edman developed chemistry for sequencing the N-terminus of a protein or a peptide[26], and his chemistry is still being used today. In the early days, the task of sequencing a protein was a significant one, limited to those proteins that could be prepared in sufficiently large amounts. Then, the aim was to obtain the full sequence of the protein in question to begin to understand how proteins were structured, which was done by using Edman chemistry exclusively. Currently, the situation is different, and it is usually necessary to obtain only partial protein sequence. Suitable oligonucleotides can be designed from the partial sequence and used to clone the corresponding gene, which can be rapidly analyzed to yield the full sequence.

Routine protein sequencing methods are based on the Edman degradation of the polypeptides, in which the N-terminal amino acid residue is specifically removed, leaving a polypeptide one residue shorter. By repeated cycles of Edman degradation and identification of product, the polypeptide can be sequenced.

In the Edman reaction (figure 1.6), the polypeptide is treated with phenylisothiocyanate (PITC), which reacts with the N-terminal amino acid residue to form a phenylthiocarbamyl (PTC) derivative of the polypeptide. Anhydrous trifluoroacetic acid (TFA) is then used to cleave the molecule, giving the 2-anilino-5-thiazolinone derivative of the N-terminal residue and the polypeptide shortened by one residue. The thiazolinone derivative is separated from the polypeptide and converted into the more stable 3-phenyl-2-thiohydantoin (PTH) derivative, which is then identified by HPLC. By repeating this cycle, the polypeptide can be sequenced from its N-terminal

end by either manual or automated techniques. In addition to being more rapid, automated sequencing is also more sensitive and capable of generating longer sequences. Cycles can be carried out with good yield (better than 95% per cycle) to give extended sequences of 50 residues or more. Edman chemistry represents the first stage of sequencing. The second is identification of the PTH-amino acid derivative produced by each cycle. Manual sequencing achieves this by thin layer chromatography, but automated sequences have on-line HPLC, so PTH-residue analysis occurs automatically. The product of each cycle of sequencing is identified by comparing it with standard PTH-amino acids.



(Figure 1.6)
Edman chemistry for N-terminal sequencing of polypeptides

4. Southern Hybridization

Southern hybridization is a commonly used technique in molecular biology to detect and identify specific nucleic acid sequences. The principles that govern molecular hybridization are used in many applications for the study of nucleic acids. Under suitable conditions, two single nucleic acid chains form a hybrid molecule to an extent that is mostly dependent on the degree of their nucleotide complementarity. The formation of such a duplex molecule occurs mainly through hydrogen bonding between guanosine and cytosine bases, and between adenosine and thymidine bases. Other base compositions are not complementary because of steric reasons and are, therefore, incompatible.

Among the various procedures that exploit molecular hybridization for the analysis of nucleic acids, perhaps the most frequently used techniques consist of the hybridization of a labeled nucleic acid probe to a target nucleic acid immobilized on a solid support such as nitrocellulose or nylon membrane. Characteristic examples of these techniques are southern and northern blotting hybridization for the analysis of DNA and RNA, respectively. In Southern-blotting hybridization[27], DNA is separated according to size by gel-electrophoresis, in-gel denatured into single-stranded molecules by treatment with alkali, neutralized, and transferred (blotted) to a hybridization membrane by capillarity using a high salt concentration buffer. DNA is then irreversibly bound to the membrane either by heat treatment or UV crosslinking. Thus, single-stranded target DNA molecules are available on the membrane for hybridization with a labeled single-stranded DNA probe. By definition, a probe is a DNA molecule with strong affinity for a specific target, and the hybrid (probe-target combination) can be revealed when an appropriate detection system is used. After removal of the nonspecific hybridization,

which would give an unacceptable high background signal, specific interactions between target and probe molecules are detected based on the type of label on the probe. Two types of labels are available: radioactive and nonradioactive.

Radioactive labels are among the most commonly used method that provide the highest degree of sensitivity and resolution currently available in hybridization assays.[28,29] However, considerations for user safety as well as cost and disposal of radioactive waste products limit the applications of radioactive probes. ^{32}P is the most widely used isotope for many reasons:

1. It has the highest specific activity;
2. It emits β-particles of high energy;
3. It does not inhibit the activity of DNA-modifying enzymes, because the structure is essentially identical to that of the nonradioactive counterpart.

The disadvantage is its relatively short half-life (14.3 d); so ^{32}P-labeled probes should be used within 1 week after preparation. The lower energy of ^{35}S, along with its longer half-life (87.4 d), make this radioisotope more useful than ^{32}P for the preparation of more stable, less specific probes. Although ^{32}S-labeled probes are less sensitive than ^{32}P, they provide higher resolution in autoradiography and are especially suitable for *in situ* hybridization procedure. Another advantage of ^{35}S over ^{32}P is that the ^{35}S-labeled nucleotides present little external hazard to the user. The lower energy β-particles barely penetrate the upper dead layer of skin and are easily contained by laboratory tubes and vials.

Similarly, ^{3}H-labeled probes (tritium) have traditionally been used for *in situ* hybridization because the low energy β-particle emissions result in maximum resolution with low background. It has the longest half-life (12.3 yr).

The use of ^{131}I has declined since the 1970s with the availability of ^{125}I-labeled nucleotide triphosphates of high specific activity. ^{125}I has the lower energies of emmision and longer half-life (60 d) than ^{131}I, and is frequently used for *in situ* hybridization.

Nonradioactive labels on the other hand have several advantages:

1. Safety;
2. Higher stability of probe;
3. Efficiency of the labeling reaction;
4. Detection *in situ*; and
5. Less time taken to detect signal.

The methods of nonradioactive labeling depend upon the use of detectable marker molecules that are covalently bound to nucleotides. These markers fall into three classes. One class is the haptens, which are detected by affinity or antibody binding; these include biotin and digoxigenin. A second class involves direct coupling of enzymes to sequences ranging in size from oligonucleotides to 50-kb fragments; enzymes commonly used for this are alkaline phosphatase (AP) and horseradish peroxidase (HRP). A third class is the fluorescent dyes, including fluorescein and rhodamine. Some comparisons between these markers and their detection techniques shown in table 1.2 below:

Table 1.2
Comparison of a number of Nonradioactive Labeling and Detection Techniques[30]

Class	Marker molecule	Incorporation method	Type of hybridization	Enzyme-coupling reaction	Detection method
I	Biotin	PCR, nick translation, end labeling	Southern, and *in situ*	Streptavidin affinity	Colorimetric, chemiluminescence, fluorescence
I	Digoxigenin	PCR, nick translation, end labeling	Southern, and *in situ*	Antibody	Colorimetric, chemiluminescence, fluorescence
II	Horseradish peroxidase	Direct coupling	Southern, and *in situ*	None	Colorimetric, chemiluminescence
II	Alkaline phosphatase	Direct coupling	Southern, and *in situ*	None	Colorimetric, chemiluminescence
III	Fluorescein, rhodamine, Texas red	PCR, nick translation, end labeling	*In situ*, DNA binding	None	Fluorescence microscopy, fluorometer

The one area in which nonradioactive probes have a clear advantage over radiolabelled probes is *in situ* hybridization. When the probe is detected by fluorescence or color reaction, the signal is at the exact location of the annealed probe, whereas radioactive probes can only be visualized as a silver grain in a photographic emulsion some distance away from the actual annealed probe.[31]

The majority of labeling procedures rely on enzymatic incorporation of a nucleotide labeled into the DNA, RNA, or oligonucleotide. Nick translation, random-primed labeling, PCR labeling, and photobiotin labeling are some of these labeling techniques.

Nick translation is one method of labeling DNA that uses the enzymes pancreatic DNase I and *Escherichia coli* DNA polymerase I. The nick translation reaction results from the process by which *E. coli* DNA polymerase I adds nucleotides to the 3'-OH

created by the nicking activity of DNase I, while the 5′ to 3′ exonuclease activity simultaneously removes nucleotides from the 5′ side of the nick. If labeled precursor nucleotides are present in the reaction, the pre-existing nucleotides are replaced with the labeled nucleotides. For radioactive labeling of DNA, the precursor nucleotide is an [α-^{32}P]dNTP. For nonradioactive labeling procedures, a biotin or a digoxigenin moiety attached to a dNTP analog is used.[29]

Random-primed labeling of DNA fragments (double or single-stranded DNA) was developed by Feinberg and Vogelstein[32, 33] as an alternative to nick translation to produce uniformly labeled probes. Double-stranded DNA is denatured and annealed with random oligonucleotide primers (6-mers). The oligonucleotides serve as primers for the 5′ to 3′ polymerase (the Klenow fragment of *E. coli* DNA polymerase I), which synthesizes labeled probes in the presence of a labeled nucleotide precursor. Like nick translation method, the random-primed method uses similar precursor nucleotides such as [α-^{32}P]dNTP for radioactive labeling and a biotin or a digoxigenin moiety attached to a dNTP analog is used for nonradioactive labeling.

A very robust method for labeling DNA is by using PCR. The gene probe is PCR-amplified using the same set of primers and thermocycling parameters; however, the dNTP mixture should have less dCTP because the labeled biotin-dCTP will also be added to the reaction. When this method is used with [α-^{32}P]dCTP, the dNTP mixture has no or low concentration of dCTP. The incorporation of the biotin molecule to the dCTP along the DNA strands during PCR cycles makes the fragment run slightly slower through the agarose gel, so a control PCR reaction without biotin should be prepared to

check the size of the desired gene. The advantage of PCR-biotin labeling is the incorporation of higher number of biotin moieties along the amplified DNA strands.

Finally, photobiotin labeling is a method that uses a chemical reaction not an enzymatic one in the labeling process. A photo-activatable analogue of biotin, N-(4-azido-2-nitrophenyl)-N'-(N-d-biotinyl-3-aminopropyl)-N'-methyl-1,3-propanediamine (photobiotin), is used for the preparation of large amounts of stable, non-radioactive, biotin-labelled DNA and RNA hybridization probes. Upon brief irradiation with visible light, photobiotin formed stable linkages with single- and double-stranded nucleic acids yielding probes. The target DNA is detected colorimetrically by avidin or streptavidin complexes with acid or alkaline phosphatase. Photobiotin labeling is the method of choice when large quantities of probe are needed and when a very high sensitivity is not needed.[34]

Once the labeled probe is prepared, the next step is to prepare an environment for the hybridization reaction to take place. Generally, this process is known as prehybridization, which involves incubation in a solution at elevated temperatures to insure the hybridization of the DNA on the surface with the labeled probe. Prehybridization and hybridization solutions are characterized by high-ionic strength, which is an important factor for hybrid stability. In addition, several blocking agents can be used to suppress nonspecific binding, thereby limiting the background signal. The main difference between the prehybridization and the hybridization solutions is the denatured probe that is added to the hybridization solution.

After hybridization the material is washed with sodium dodecyl sulfate (SDS)/SSC solution to remove any unbound material. In many cases, the hybridization

solution can be saved and reused. Generally, after this step the material is washed in a buffer to remove the washing solution and to prepare the material for the blocking reagent. The blocking buffer is used to reduce the nonspecific binding of the conjugate. The material is then treated with a solution containing the affinity molecule (avidin) or antibody (antidigoxigenin) conjugated to a reporter enzyme (AP or HRP). A washing step follows to remove the unbound reporter conjugate. Finally, the material is soaked in a substrate on which the reporter enzyme will act. These molecules include Nitro blue tetrazolium (NBT) and 3,3'-diaminobenzidine (DAB), which are colorimetric substrates for AP and HRP, respectively.[35,36] As was mentioned before, nonradioactive methods have the speed advantage over the radioactive counterpart. As you can see from table 1.3, at the most, two sets of hybridization can be produced a week with radioactive probes. With nonradioactive probes, it is possible to do hybridization on one day and have the data ready to be analyzed the next afternoon.

Table 1.3
Comparison of Timing Protocols of Nonradioactive Detection Methods[30]

#	Method	Hybridization Time	Wash Time	Exposure Time
1	Radiation	Overnight	1 hour	1-2 days
2	Biotin or Digoxigenin (colorimetric)	Overnight	3 hours	Instantaneous
3	Enzyme labeled oligonucleotides	15 min	30 min	3 hours
4	Fluorescent *in situ*	Overnight	15 min	Instantaneous

There are a few drawbacks to using nonradioactive methods, although these are greatly outweighed by the advantages. The first is that the reagents are fairly sensitive to quality of preparations and cleanliness of glassware; these problems are easily solved by careful

handling of reagents and glassware. The second is that the target materials must be handled carefully; the sensitivity of this technique means that the scratches in the surface are visible, and fingerprints will show up as dark smudges and can never be removed from the surface.

5. Statement Of Purpose

This thesis proposal describes the purification and characterization of threonine dehydrogenase from *Klebsiella pneumoniae* and *Enterobacter aerogenes*. In addition, the amino terminal sequence of each protein will be determined and compared to that of other dehydrogenases in a phylogenetic tree generated using the Wisconsin Package of the Genetic Computer Group.

CHAPTER II
MATERIALS

2.1 Growth of Klebsiella *pneumoniae* & Enterobacter *aerogenes*

- *Klebsiella pneumoniae subsp. Rhinoscleromatis* was purchased from American Type Culture Collection®, ATCC # 13884.
- *Enterobacter aerogenes* stock was a generous gift of Professor Jeff Lodge from the Rochester Institute of Technology Dept. of Biological science.
- Yeast extract from Becton Dickinson.
- Bacto®-Tryptone from DIFCO Laboratories.
- Levine EMB agar from DIFCO Laboratories.
- Sodium Chloride from J.T.Baker.
- Glycerol from J.T.Baker.

2.2 Enzyme Purification

- Poros 50 DEAE weak anion exchange packing from PerSeptive Biosystems.
- Cibracron Blue F3GA from Sigma.
- TOYOPEARL® HW-55S from Superlco, Inc.
- Centriplus® Concentrators by Amicon.
- Acrodisc® PF 0.22 μm and 0.45 μm, by Gelman Sciences.

2.3 Electrophoresis

2.3.1 SDS-PAGE

- Ammonium persulfate, from J.T.Baker.
- Sodium dodecyl sulfate (SDS) from Sigma.
- Tris-base from J.T.Baker.
- 30% acrylamide/Bis solution from Bio-Rad Laboratories.
- TEMED from Bio-Rad Laboratories.
- Glycine from Bio-Rad Laboratories.
- Bromophenol blue from Bio-Rad Laboratories.
- Coomassie blue stain from Bio-Rad Laboratories.
- Low range molecular weight standards from Bio-Rad Laboratories.

2.3.2 Agarose Gel Electrophoresis

- Standard low electroendosmosis agarose from J.T.Baker.
- Tris-acetate from J.T.Baker.
- Sodium acetate from J.T.Baker.
- 1 kb Ladder from New England Biolabs, Inc.
- Loading Dye from Carolina Biologicals, Inc.
- Ethidium Bromide from Bio-Rad Laboratories.

2.4 Molecular Weight Estimate

Gel filtration standard was purchased from Bio-Rad Laboratories.

2.5 Protein and Activity Assays

- Protein assay dye reagent concentrate from Bio-Rad Laboratories.
- Bovine gamma-globulin from Worthington Biochemical Corporation.
- L-Threonine from J.T.Baker.
- β-NAD^+ from Sigma.
- Phenazine methosulfate, (PhMS) from Sigma.
- p-iodotetrazoliumviolet, (pITV) from Sigma.

2.6 DNA Isolation

2.6.1 Genomic DNA

- Sorbitol from J.T.Baker.
- Dithiothreitol from Sigma
- Lyticase from Sigma
- Potassium acetate from J.T.Baker
- Ethanol from AAPER Alcohol and Chemical CO.
- RNase from QIAGEN
- Sodium acetate from J.T.Baker.
- 2-Propanol from J.T.Baker.

2.6.2 Plasmid DNA

QIAprep Spin Miniprep Kit was ordered from QIAGEN. Reagents provided: RNase A, buffer P_1, buffer P_2, buffer N_3, buffer PB and buffer PE.

2.7 Polymerase Chain Reaction

- Pfu Polymerase was a generous gift of Dr. David Lawlor from the Rochester Institute of Technology Dept. of Biological science.
- dNTP Mix from Stratagene.
- 10x Cloned Pfu Polymerase Buffer, from Stratagene.
- Magnesium Chloride from Perkin Elmer.
- *E. coli* primers were synthesized at University of Rochester Core Nucleic Acid Laboratory.

2.8 QIAquick Gel Extract

QIAquick gel extract kit was purchased from QIAGEN; the reagents provided are: Buffer QG, Buffer PE, Buffer EB, QIAquick spin column, and collection tubes (2 mL).

2.9 Nonradioactive Nucleic Acid Labeling and Detection

2.9.1 Preparation of Biotin-labeled DNA Probes by random oligonucleotide-primed synthesis

- Biotinylated random octamers from New England Biolabs, Inc.
- dNTP/biotin mix from New England Biolabs, Inc.
- Klenow fragment from New England Biolabs, Inc.
- EDTA and lithium chloride from J.T.Baker

2.9.2 BlueGene detection method

- Pure nitrocellulose membrane (0.45 μM) from BioRad Laboratory.
- SA-AP conjugate from GibcoBRL.
- Nitroblue tetrazolium (NBT) from Sigma.
- 5-bromo-4chloro-3-indolylphosphate (BCIP) from Sigma.
- Salmon sperm DNA from GibcoBRL.
- Magnesium chloride from J.T.Baker.
- Sodium dodecyl sulfate From Sigma
- Ficoll from Sigma.
- Polyvinylpyrrolidone from Sigma.
- Bovine Serum Albumin (fraction V) from Sigma.
- Formamide from Fluka Biochemika
- Dextran sulfate from Sigma.

2.10 Instrumentation

Instrumentation used for experiments were from the sources listed:

- Waters™ 650 protein purification system and 486 tunable absorbance detector from Waters.
- Fraction collector (model FC 205) from Gilson.
- Multistatic® pump, by LABCONCO.
- SORVALL® RC 5C Plus centrifuge with SLA-1500 and SA-600 rotors were purchased from Dupont.
- Desktop Biofuge 13 from Heraeus Instruments.
- Fisher Micro Centrifuge, model 235A from Fisher Scientific.
- Sonifier® sonicator from Branson Instruments, Inc.
- GeneAmp PCR System 2400 from Perkin Elmer.
- UV-VIS spectrophotometer model 8453 from Hewlett Packard.
- MRX Microplate reader from DYNEX Technologies.
- Spectronic 1201 by Milton Roy.
- Electrophoresis assemblies and electrophoresis power supply (model PAC 300) from Bio-Rad Laboratories.
- 37° C water bath model 1265PC, 37° C incubator model 1545, and -85° C freezer from VWR Scientific.
- Fisher HI-TEMP™ BATH model 160.
- Laboratory Vacuum sterilizer, Novus I, by Getinge
- DC40 camera from Kodak Digital Science.
- Transilluminator model FBDLT-88 from Fisher Scientific.

2.11 General chemicals

General chemicals used routinely were reagent-grade products of the companies indicated:

Sodium hydroxide, potassium chloride, hydrochloric acid, methanol, acetic acid, and sodium azide from J.T.Baker; 2-mercaptoethanol from Sigma; ethanol from AAPER Alcohol and Chemical CO.

CHAPTER III
METHODS

3.1 Protein Purification

Threonine dehydrogenase was purified from two different types of bacteria; *Klebsiella pneumoniae* and *Enterobacter aerogenes*. Similar steps were taken to purify the protein from the crude extract of the two bacteria. Therefore, the protocol outline of the purification and analysis steps will refer to both organisms unless stated otherwise.

3.1.1 Bacterial Growth

The bacterial stock cells were grown in Luria Broth (LB) media, diluted with an equal volume of sterile glycerol, and stored at –85° C. One aliquot was thawed and maintained at –20° C for later use. LB media was prepared [29] by dissolving 10 g NaCl, 5g bacto-yeast extract, and 10 g bacto-tryptone in one liter of deionized water and sterilized by autoclaving. LB agar plates were prepared by adding 15g/liter bacto-agar to the liquid LB medium was prepared according to the recipe given above, before autoclaving. After autoclaving, the media was allowed to cool to 50° C before pouring to plates. Plates of Levine EMB agar were prepared by dissolving 37.5g of the media premix in a liter of deionized water and sterilized by autoclaving.

5 mL of LB media was inoculated with a single bacterial colony previously grown on LB media plate. The 5 mL LB media was incubated in a 37° C shaker for 10-16 hours then transferred to a 500 mL LB flask. The solution was vigorously shaken at 37° C overnight. The bacterial cells were harvested by centrifugation at 10,000xg, (8,600 rpm) for 10 min using RC-5C Plus centrifuge equipped with the SLA 1500 rotor. Supernatant

was decanted and the cell paste was transferred to a 50 mL conical centrifuge tube and stored at –20° C. The wet weight of the cells was recorded.

3.1.2 Preparation Of Crude Extract

The bacterial cells were resuspended (2 mL buffer/1 g of cells) in cold 0.05 M Tris-HCl, pH 8.4 (0.5 mM 2-mercaptoethanol and 0.02% NaN_3 were added to all buffers). Sonic oscillation (2 min burst/5 g of cells) was the method used to fracture *K. pneumoniae*'s capsule and *E. aerogenes* cell wall. The cells were kept on ice to maintain the temperature at or below 10° C. The resulting homogenate was centrifuged at 20,000xg (11,500 rpm) for 30 min. The supernatant was filtered by Acrodisc 0.45 μm then with Acrodisc PF 0.22 μm filter and stored at 4° C. A 0.5 mL aliquot was saved in the –20° C freezer for later analysis.

3.1.3 Ion-Exchange Method

A Poros 50 DEAE weak anion exchange packing from PerSeptive Biosystems was packed in a (1 cm ID X 60 cm) column and used for the first purification step. For each run, 5 mL of the crude extract was injected onto the column. The program gradient for Waters™ 650 protein purification system was set as shown in the table below:

Time (min)	Flow(mL/min)	%A	%B	%C	%D
initial	4	6	14	0	80
2	4	6	14	0	80
4	4	6	14	5	75
8	4	6	14	10	70
10	4	6	14	10	70
14	4	6	14	15	65
16	4	6	14	15	65
18	4	6	14	20	60
24	4	6	14	80	0
50	4	6	14	80	0
50:01	0	6	14	80	0

1. Reservoir A is a 100 mM Tris-HCl buffer
2. Reservoir B is a 100 mM Tris base buffer
3. Reservoir C is 2 M KCl
4. Reservoir D is nanopure water

The fractions were collected (1 min/tube) and assayed for TDH activity (see section 3.3). The final fractions were in a 40 mM Tris buffer (pH 8.5). A 0.5 mL aliquot was saved in the –20° C freezer for later analysis.

3.1.4 Dye Affinity Method

A dye-ligand affinity chromatography column was prepared by gravity packing Cibacron Blue 3GA (Reactive Blue 2 Sepharose) into a (4 cm ID X 24 cm) column that was connected directly to the fraction collector, and the buffer flow was based on gravitational force. The ion exchange pool (~8 mL) was applied to the column at (pH 7.0). Then the column was washed with 600 mL of 20 mM Tris buffer (pH 7.0). In order to elute the enzyme, 100 mL of 20 mM Tris buffer (pH 8.5) containing 5 mM NAD^+ was washed through the column. TDH activity was assayed by colorimetric microplate assay and active fractions were pooled together. The pool was concentrated and a 0.5 mL aliquot was saved at –20° C for later analysis.

3.1.5 Gel Filtration Method

A gel filtration column was prepared by gravity packing TOYOPEARL® HW-55S media into a Waters AP-1 (1 cm ID X 60 cm) column, which was connected to a Water 650E Protein Purification System. Before applying the sample to the column, the fraction pool from Cibacron Blue F3GA was concentrated using AMICON CENTRIPLUS® Concentrators centrifuged at 3000xg (4,600 rpm) for 35 min. The

running buffer used for this separation was 20 mM Tris pH 8.5 containing 0.1 M KCl. The flow rate was set at one mL/min. Threonine dehydrogenase activity was determined using the colorimetric activity assay. Active fractions were pooled together and concentrated to a final volume of 0.75 mL.

3.1.6 SDS-PAGE Electrophoresis

Polyacrylamide gel electrophoresis was carried out to determine the homogeneity of samples saved from each purification step. The samples were diluted with an equal volume of 2x Laemmli buffer (1 mL 0.5 M Tris-HCl (pH 6.8) solution: 4 mL distilled water, 0.8 mL glycerol, 1.6 mL 10% SDS, 0.4 mL 2-mercaptoethanol and 0.2 mL of 0.5% bromophenol blue). The gel consisted of a 10% acrylamide separating gel and a 4% stacking gel. Electrophoresis was carried out in (0.3% Tris base, 1.45% glycine, 0.1% SDS, pH 8.0) running buffer at 120 volts for about 70 min until the tracking dye reached the bottom of the gel. The gel was stained in Coomassie Blue stain solution which consisted of 40% methanol, 10% acetic acid, 50% ddH_2O and 0.1% (w/v) Coomassie Blue. After 20 minutes of staining, the gel was destained in 40% methanol, 10% acetic acid, and 50% water over night or until Coomassie blue stain was gone. Relative migration (R_m) was determined by dividing the migration distance of the protein bands by that of the tracking dye.

3.2 Molecular Weight Estimate

Using the molecular weight standards that were purchased from Bio-Rad, the TDH molecular weight was estimated by running the pure enzyme on the gel filtration column with a flow rate of 0.5 mL/min and a duplicate run was done using the standards as a reference point. The relative migration (R_m) was calculated, compared with the

standards, and used to estimate the MW of the native protein. SDS-PAGE was another method that was used to estimate the MW of TDH. Similar to the gel filtration method, the SDS-PAGE method uses a plot of Log of MW versus R_m of standards to make a standard curve. Then, MW of the TDH subunit can be estimated from the standard curve.

3.3 Enzyme Activity

Threonine dehydrogenase activity was assayed using the following two methods:

A) Colorimetric Microplate Assay: This assay was done routinely to detect TDH activity throughout the purification process.[37] The assay solution consisted of 0.2 M Tris-HCl (pH 8.4), 0.125 M threonine, 5 mM NAD^+, 5 μg/mL phMS, and 200 μg/mL pITV. 150 μL assay solution was added to wells in a microtiter plate that contained 30 μL of the TDH samples. Then a microplate reader followed the A_{490} for 5 minute. Activity was also detected by visual inspection of the pink color produced in the presence of threonine dehydrogenase activity.

B) Spectrophotometric Assay: The main principle of this assay is the formation of NADH at A_{340} nm due to the enzymatic oxidation of threonine. This assay was used to study the aliquot samples saved after each purification step and crude extract to determine the specific and total activity of the enzyme. The assay solution consisted of 0.2 M Tris-HCl (pH 8.4), 0.125 M threonine, and 5 mM NAD^+. Then, 1 mL of assay solution was added to each of the disposable polystyrene cuvettes containing enzyme solution (10-50 μL) and linear reaction rates were observed for the first 2-3 minutes at 37° C. A series of enzyme dilutions was assayed to ensure that the rate of the reaction is proportional to enzyme concentration. Finally, the rate of the reaction was calculated using 6.22×10^3 for the molar extinction coefficient[38] of NADH.

3.4 Bradford Dye-Binding Assay

Protein concentration in solution was determined using the Bio-Rad version of the Bradford dye-binding assay.[39] Bovine gamma globulin served as standard and a calibration curve was prepared. The Dye Reagent Concentrate was diluted 1:4 with ddH_2O and filtered through a Whatman #1 filter paper to remove solid particles. Crude extract and aliquot samples saved from each purification step were diluted with water to a total volume of 120 μL in all tubes. Then 5 mL of diluted dye reagent was added to each tube and vortexed well. The mixtures were incubated at room temperature for 5 min. Absorbance values at 595 nm were measured for all the samples using a disposable cuvette by a UV-Visible spectrophotometer. The concentration of the TDH for each sample was determined based on the calibration curve.

3.5 Protein Sequencing

Protein sequencing and the sequence data were generously provided by Michael Wetherell from Wyeth-Lederle Vaccines.

3.6 DNA Isolation

In order to sequence the TDH gene from *Klebsiella pneumoniae,* it was necessary to isolate its genomic DNA. The *E. coli* plasmid (SBD76-pDR121) was also isolated to be used as a control in the PCR amplification of the TDH gene from *Klebsiella pneumoniae* genome.

3.6.1 Isolation of *Klebsiella pneumoniae* Genomic DNA

This method is designed to isolate genomic DNA from 30 mL LB bacterial cell culture. Cells were collected by centrifuging at 5,000 rpm for 5 min followed by washing the pellet in 10 mL DNase free ddH2O. The pellet was resuspended in 3 mL of buffer

(0.9 M sorbitol, 0.1 M EDTA, 50 μM dithiothreitol, pH 7.5). To break the cell wall, 0.5 mg lyticase was added to 200 μL 0.9 M sorbitol and incubated in 37° C shaker for 3 hours.

Spheroplasts were collected at 5,000 rpm for 5 min before discarding the supernatant. The pellet was resuspended in 3.0 mL (50 mM Tris-HCl, 50 mM EDTA, pH 8.0) and 0.3 mL 10% SDS was added to solubilize the cell membrane at 65° C for 30 min. 1.0 mL of 5M potassium acetate was added and the solution was kept on ice for 60 min. Then, the solution was spun at 15,000 rpm for 30 min and supernatant was transferred to a previously autoclaved polytube. DNA/RNA were washed with 4.0 mL ice-cold 95% ethanol then with 50% ethanol. In each wash, the DNA was collected by spinning at 10,000 rpm for 10 min and carefully discarding the supernatant; DNA was in the bottom of the polytube. After the last wash, the pellet was dried at 50° C for ~ 4 hours (or can be left to dry at room temperature overnight) then rehydrated in 3.0 mL (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). A 0.5 mL aliquot was saved to evaluate the ratio of DNA to RNA by gel electrophoresis.

After the release of the DNA from the cells, RNA can be removed by treating the solution with RNase (150 μL of 1.0 mg/mL RNase in 10 mM NaOAc). The solution was incubated for 30 min at 37° C. The RNA residues were washed twice, with 4.0 mL ice-cold 95%, and 50% 2-propanol. Finally, the pellet was resuspended in 0.5 mL (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The genomic DNA size and purity was checked with agarose gel electrophoresis. DNA purity and concentration was determined from the ratio of A_{260}/A_{280} nm. A ratio of 1.8-2.0 suggests minimal protein contamination.

3.6.2 QIAprep Plasmid Preparation Kit

The QIAprep Plasmid Preparation Kit[40] was used for *E. coli* plasmid (SBD76-pDR121) isolation. All centrifugation steps for the miniprep were performed on a Desktop Biofuge 13 microfuge at maximal speed (10,000 x g or 13,000 rpm).

Cells were centrifuged at 13,000 rpm for 5 min. Then, cell pellets were resuspended in 250 μL buffer P1. 250 μL of buffer P2 was added to each tube and gently inverted 4-6 times to mix. In buffer P2, the bacterial cells were lysed in sodium hydroxide, SDS and RNase A. SDS was used to solubilize the phospholipid and protein components of the cell membrane, which lysed and released the cell components while the alkaline conditions denatured the chromosomal, and plasmid DNAs as well as proteins. The lysate was neutralized and adjusted to high salt binding conditions by adding 350 μL of buffer N3. The tubes were inverted immediately but gently 4-6 times until the solution became cloudy and very viscous. All the tubes were centrifuged for 10 min and a compact white pellet formed. The high salt concentration causes the denatured proteins, chromosomal DNA, cellular debris, and SDS to precipitate, while the shorter plasmid DNA renatures correctly and stays in solution. The QIAprep spin columns were placed in 2 mL collection tubes and the clear lysate was loaded onto the QIAprep column assemblies. They were centrifuged for 60 sec and the filtrates were discarded. The optimized buffer conditions in the lysate and the unique silica-gel membrane ensured the only DNA will be adsorbed, while RNA, cellular proteins, and metabolites are not retained on the membrane and are found in the flow-through. All the salts were washed away with 0.75 mL of buffer PE for each QIAprep spin column, followed by centrifuging at top speed for 60 sec. QIAprep spin columns were centrifuged for an additional 1 min

to remove residual wash buffer. Then, QIAprep spin columns were placed in clean 1.5 mL microfuge tubes and 50 μL nanopure and autoclaved water was added. The assemblies were centrifuged for 1 min after standing at room temperature for 1 min. The filtrates containing the plasmids were collected in separate microfuge tubes. An aliquot was analyzed on 0.8% agarose gel and the rest was stored at –20° C for later PCR analysis.

3.7 Polymerase Chain Reaction (PCR)

This protocol was carried out using the GeneAmp PCR system 2400. The PCR reaction mix consisted of the following reagents:

1 μL Pfu polymerase (2.5 U)
1 μL dNTP mix (0.1 mM)
3 μL of 10x Pfu Buffer
2 μL of $MgCl_2$ (2.5 mM)
3 μL of each primer (3.2 pmole)
2 μL double-stranded plasmid DNA (0.4 μg)
Autoclaved ddH_2O to a total volume of 30 μL.

The following program was cycled 25 times and kept at 4° C until removal.

Temperature	Time		
95° C	10 seconds	Initiation step	
95° C	10 seconds	Denaturation	Repeated 25 cycles
40° C	3 minute	Annealing	
67° C	4 minutes	Elongation	
72° C	7 minutes	Polish ends	
4° C	until removal		

The results were checked on 0.8% agarose gel electrophoresis followed by gel extract of the gene (see section 3.9).

3.8 Agarose Gel Electrophoresis

0.8% agarose gel was prepared in TAE buffer (40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA, pH 8.0). A 1kb ladder was used as standard. The gel was run at 120 Volts for about 75 min until the tracking dye approached the end of the gel. Then the gel was stained in 0.2 μg/mL ethidium bromide for 15 min and destained with distilled water. A picture of the gel was taken on the UV light box by Kodak digital camera, (Model DC40). The picture was analyzed and printed using Kodak Digital Science 1D software.

3.9 QIAquick Gel Extract

The QIAprep Gel Extraction Kit[41] was used to extract the DNA from agarose gels. The DNA fragment was excised using a clean sharp scalpel. Next, 3 volumes of buffer QG was added to 1 volume of gel (100 mg of gel slice ~ 100 μL of volume). Buffer QG solubilizes the agarose gel and provides the appropriate conditions for binding the DNA to the silica membrane of the column. The mixture was incubated at 50° C until the gel slice was completely dissolved. The sample was applied to the QIAquick spin column and was centrifuged at 13,000 rpm for 1 min. The DNA was bound to the column and the flow-through was discarded. Then, salts were washed away by 0.75 mL ethanol-containing buffer PE. The solution was centrifuged for 1 min, followed by another centrifuging for an additional minute. Finally, the QIAquick column was placed in a 1.5 mL microfuge tube, and the DNA was eluted with 40 μL of autoclaved ddH_2O by centrifuging at top speed (13,000 rpm). The DNA concentration and purity were determined by A_{260}/A_{280} ratio.

3.10 Cycle Sequencing

The protocol was taken from the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit.[42] Each sequencing reaction consisted of 8.0 μL Terminator Ready Reaction Mix, 0.4 μg double-stranded plasmid DNA, 3.2 pmole primer, and ddH_2O to a total volume of 20 μL. The reaction was carried on the GeneAmp PCR system 2400. The sequencing primers were synthesized at University of Rochester Core Nucleic Acid Laboratory and are the same as the gene amplification primers, and they are:

(031) 5'-ACGCGTGCAGTAGAAGCAATTTACGCG-3';

(030) 5'–CGCGTGCAGTAGAAGC ATTTACGCG-3'.

(636) 5'–GTCGTGGGCCATGAATATGT-3'

(103) 5'–CGATAGACATATCAGACGGC-3'.

The following program was cycled 25 times and kept at 4° C until removal.

Temperature	Time		
96° C	10 seconds	Denaturation	Repeated 25 cycles
40° C	5 seconds	Annealing	
60° C	4 minutes	Elongation	
4° C	until removal		

The reaction tubes were sent to University of Rochester Core Nucleic Acid Laboratory for DNA sequencing.

3.11 Preparation Of Biotin-Labeled DNA Probes

TDH biotinylated probe was generated using the following two methods:

3.11.1 Random Oligonucleotide-Primed Synthesis

In order to prepare the TDH probe, 0.5-2.0 μg of the *E. coli* DNA was placed in a microcentrifuge tube and nuclease-free water was added to a total volume of 39 μL. The DNA was denatured by boiling for 5 min followed by placing it on dry ice for 5 min. The following materials were added to the sample in the order listed:

1. 5 μL of 10x biotinylated random octomers.
2. 5 μL dNTP/biotin mix (0.5 mM in Tris-HCl, pH 7.0).
3. 1 μL Klenow fragment (5 units/μL).

An additional 2.5 U of Klenow fragment was added after 30 min of the incubation at 37° C to boost the reaction. The reaction was incubated for up to 6 hours and terminated by adding 3 μL of 0.5M EDTA, pH 8.0. The resulting probe was precipitated by adding 5 μL of 4 M LiCl and 150 μL ice-cold 100% ethanol. The mixture was allowed to cool by placing it on dry ice for 30 min. Finally, the solution was microcentrifuged for 10 min at top speed and at room temperature, followed by washing the DNA pellet with 15 μL ice-cold 70% ethanol. The final DNA pellet was resuspended in 20 μL TE buffer, pH 7.5 and stored at -4°.

3.11.2 PCR Method For Generating A Biotinylated Gene Probe

This method was done by repeating the PCR steps used for the amplification of the TDH gene (section 3.7) except for using biotinylated cytosine nucleotides to label the resulting gene and hence be used as a probe for maximal specificity.

3.12 Colorimetric Detection Of Biotinylated Probe

In order to identify the sensitivity as well as the specificity of the probe and to test its effectiveness in identifying the TDH gene from *E. coli* as well as from *K. pneumoniae* the reactions were set up as follows:

Using dilution buffer, (1 M NaCl, 0.1 M sodium citrate, pH 7.5) the DNA was diluted to the following concentrations: (1, 2, 5, 10, 20, 40, 80, and 160) pmol/μL. Then, each dilution was spotted on a nitrocellulose membrane. Later, the membrane was dried in a vacuum oven at 80° C. After 1-2 hours in the oven, the membrane was rehydrated in 2x SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.5).

3.12.1 Hybridization

Prehybridization solution (20-100 μL of prehybridization solution/cm^2 of membrane) is prepared by mixing the following reagents: [50% formamide, 5x SSC, 5x Denhardt's solution (1% w/v of each of the following: Ficoll, polyvinylpyrrolidone, BSA fraction V), 20 mM sodium phosphate (pH 6.5), 0.5 mg/mL freshly denatured sheared salmon sperm DNA]. The membrane was incubated at 42° C in sealed polypropylene bag for 2-4 hours.

Hybridization solution is prepared as follows: [45% formamide, 5x SSC, 1x Denhardt's solution, 20 mM sodium phosphate (pH 6.5), 0.2 mg/mL freshly denatured sheared salmon sperm DNA, 5% dextran sulfate, 0.1-0.5 μg/mL freshly denatured probe DNA]. The probe (100 ng/mL) as well as the salmon sperm DNA was heat-denatured just before hybridization. (20-100 μL/cm^2) of hybridization solution was added after removing the prehybridization solution. The polypropylene bag was resealed after

extruding air bubbles from it and the membrane was hybridized at 42° C overnight for maximal sensitivity.

3.11.2 Post hybridization washes

After hybridization, the membrane was washed with the following solutions:

a. 250 mL of 2x SSC/0.1% SDS for 3 min at room temperature. Repeat once.

b. 250 mL of 0.2x SSC/0.1% SDS for 3 min at room temperature. Repeat once.

c. 250 mL of 0.16x SSC/0.1% SDS for 15 min at 50° C. Repeat once.

d. The membrane was briefly washed with 2x SSC at room temperature.

3.11.3 Membrane Blocking

Membrane was washed with alkaline phosphatase buffer [0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl] for 1 min; then, incubated for 1 hour at 65° C in 3% Bovine Serum Albumin fraction V (3g BSA/100 mL AP buffer).

3.11.4 Conjugate Binding

Immediately prior to use, 7 µL of SA-AP conjugate [1 mg/mL in 3 M NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 30 mM triethanolamine (pH 7.6)] was diluted to 7 mL of AP buffer (pH 7.5) for each 100 cm^2 of membrane. The membrane was incubated for 10 min at room temperature with gentle agitation. After the short incubation, the membrane was washed twice with 140-280 mL of AP (pH 7.5) for 15 min at room temperature. This wash was repeated one more time with AP buffer (pH 9.5) for 10 min at room temperature.

3.11.5 Visualization

The dye solution was prepared freshly prior to use by gently mixing 33 µL nitro blue tetrazolium (NBT, 75 mg/mL in 70% dimethylformamide) to 7.5 mL AP buffer (pH

9.5). Then, 25µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 50 mg/mL in dimethylformamide) solution was added followed by gentle mixing. The membrane was incubated with dye solution in a dark or low light for (0.5- 3) hours. Longer incubations resulted in increased background. After the incubation, the membrane was washed in TE (20 mM Tris-HCl (pH 7.5), 0.5 mM Na_2EDTA) buffer to terminate the color development reaction. The membrane was dried by baking at 80° C for 1-2 min.

CHAPTER IV
RESULTS

4.1 Protein purification and molecular weight determination

Sequencing the TDH gene from *K. pneumoniae* and *E. aerogenes* was the main goal for this thesis project. Two different approaches were taken to achieve this goal. The first was to determine the amino terminus sequence of the native protein. Threonine dehydrogenase was reproducibly obtained in homogeneous form from extracts of *K. pneumoniae* and *E. aerogenes* cells using three chromatography steps: DEAE ion exchange, Blue Dextran-Sepharose, and Gel Filtration.

Figure 4.1 shows the elution profile of DEAE column for *K. pneumoniae*'s cell extract. 5 mL of crude extract with approximately 3.49 mg of protein was loaded on the ion-exchange column with a flow rate of 4 mL/min and buffer 20 mM Tris pH 8.5 with salt gradient ranges from 0-1.6 M KCl. TDH activity was detected at fractions 23, and 24 when the salt concentration was 1.35 M.

Figure 4.2 shows the elusion profile of DEAE column for *E. aerogenes*'s cell extract. 5 mL of crude extract with approximately 4.18 mg of protein was loaded on the ion-exchange column with a flow rate of 4 mL/min and buffer 20 mM Tris pH 8.5 with salt gradient ranges from 0-1.6 M KCl. TDH activity was detected at fractions 24 and 25 when the salt concentration was 1.4 M.

Both fractions from the DEAE column were then pooled and loaded on to the prepared Cibacron Blue 3GA affinity column and eluted with 100 mL buffer (0.02 M Tris, pH 8.5) with 5 mM NAD^+. *K. pneumoniae* fractions from the affinity column were

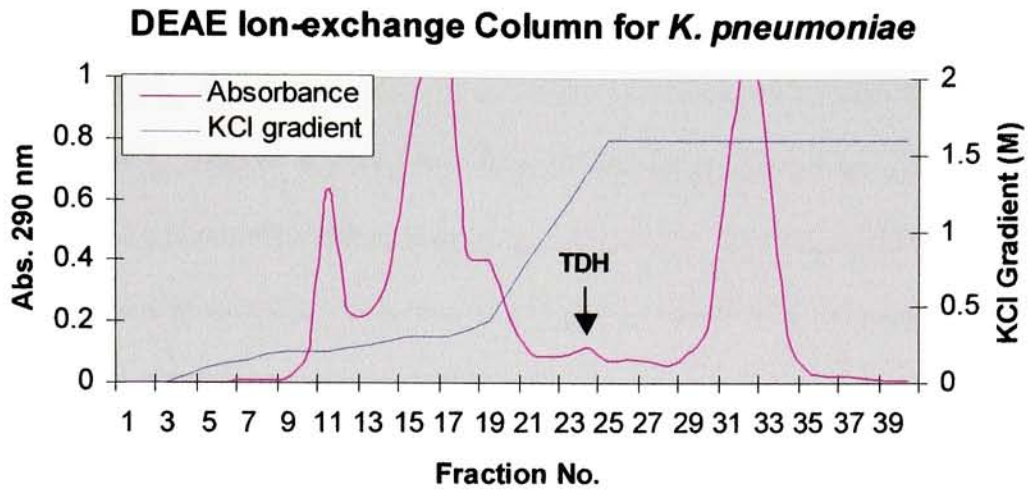


Figure 4.1 Elution profile for TDH activity from DEAE ion-exchange column. Injection volume: 5 mL, Flow rate: 4 mL/min, Buffer: 20 mM Tris pH 8.5 with KCl gradient. Detector wavelength at 290 nm, AUFS 2.0. Fraction collector: 1 mL/tube. TDH activity was detected at fractions 23, and 24 when salt concentration was 1.35 M.

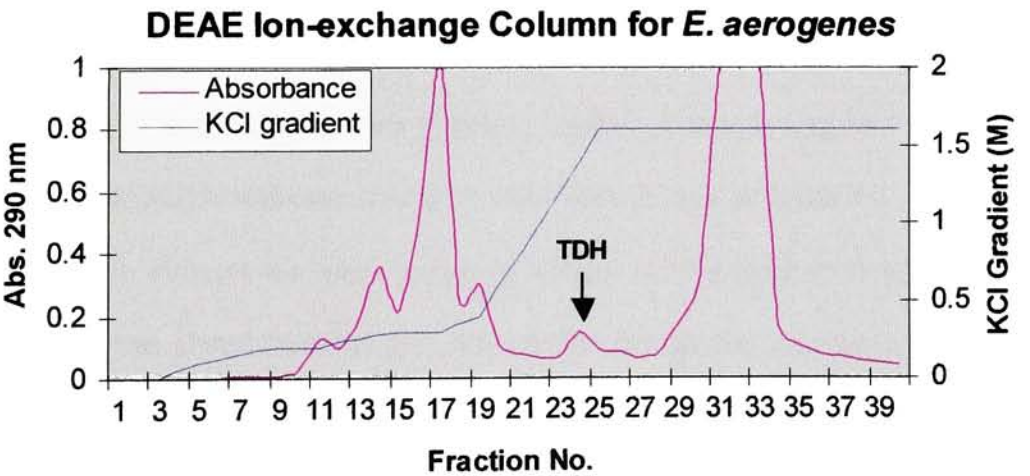


Figure 4.2 Elution profile for TDH activity from DEAE ion-exchange column. Injection volume: 5 mL, Flow rate: 4 mL/min, Buffer: 20 mM Tris pH 8.5 with KCl gradient. Detector wavelength at 290 nm, AUFS 2.0. Fraction collector: 1 mL/tube. TDH activity was detected at fractions 24, and 25 when salt concentration was 1.4 M.

pooled and concentrated to a final volume of 3 mL and 0.12 mg total protein. As for *E. aerogenes*, the final concentration was 2 mL with a total protein of 0.04 mg. The concentrated TDH from the affinity column were then loaded on the Toyopearl HW-55s gel filtration column. At a flow rate of 1.0 mL/min and a buffer of 20 mM Tris pH 8.5 with 100 mM KCl. Figures 4.3, and 4.4 show the elution profile of the gel filtration column for both *k. pneumoniae* and *E. aerogenes* respectively. The active fractions from this method were pooled and concentrated to a final volume of 0.75 mL and a protein concentration and activity assays were preformed as described in chapter 3 to determine the final protein concentration and the specific activity as presented in tables 4.1, and 4.2.

Figure 4.5 is an SDS-PAGE gel for *K. pneumoniae* TDH. The gel clearly shows the protein homogeneity in lanes A, B, and C. Using the molecular weight standards in lane E, the TDH molecular weight was estimated by plotting the log of standards molecular weight versus the relative migration (R_m) see figure 4.6. An estimated subunit MW for *k. pneumoniae* TDH was calculated to be (38.8kDa)

Similar steps were taken with E. aerogenes. In figure 4.7, SDS-PAGE gel shows the homogeneity of the purified protein in lanes F, and G. Using the log MW vs. R_m the subunit molecular weight was estimated to be (43.1 kDa) as seen in figure 4.8.

In order to estimate the native molecular weight and the number of subunits, the purified protein was eluted from the gel filtration chromatography column in Toyopearl HW-55s resin at (45 min) for *K. pneumoniae* and (49 min) for *E. aerogenes* as seen in figures 4.10 and 4.11 respectively. This elution time difference between the two proteins clearly indicated that the MW of *K. pneumoniae*'s TDH is larger than that of *E. aerogenes*. Using the MW standards purchased from Bio-Rad Laboratories which

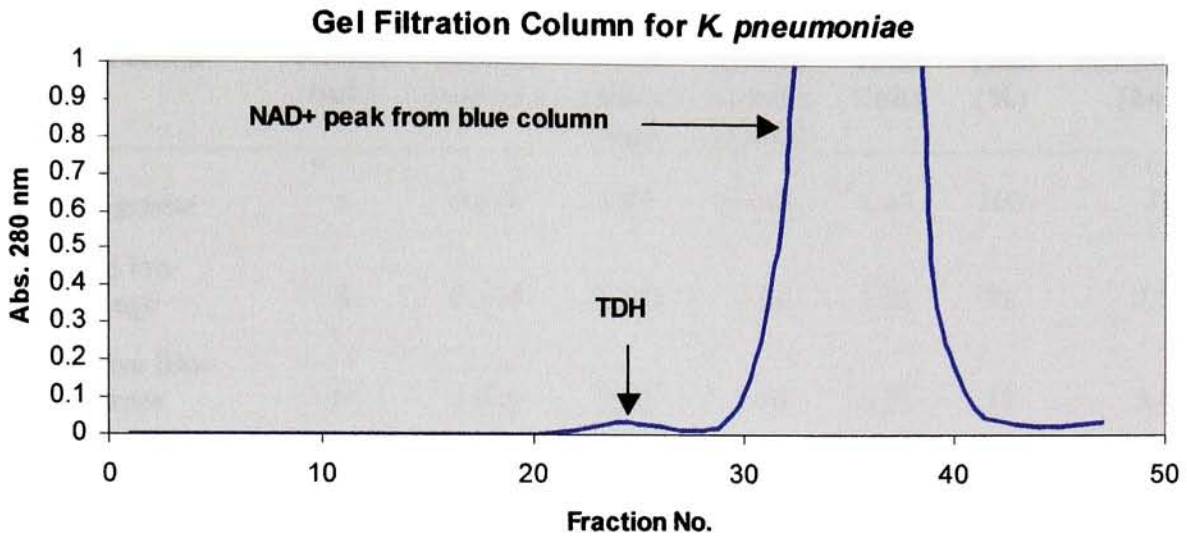


Figure 4.3 Elution profile for TDH activity from gel filtration column. Injection volume: 2 mL, Flow rate: 1 mL/min, Buffer: 20 mM Tris pH 8.5 with 100 mM KCl. Detector wavelength at 280 nm, AUFS 1.0. Fraction collector: 1 mL/tube. TDH activity was detected at fractions 23-26.

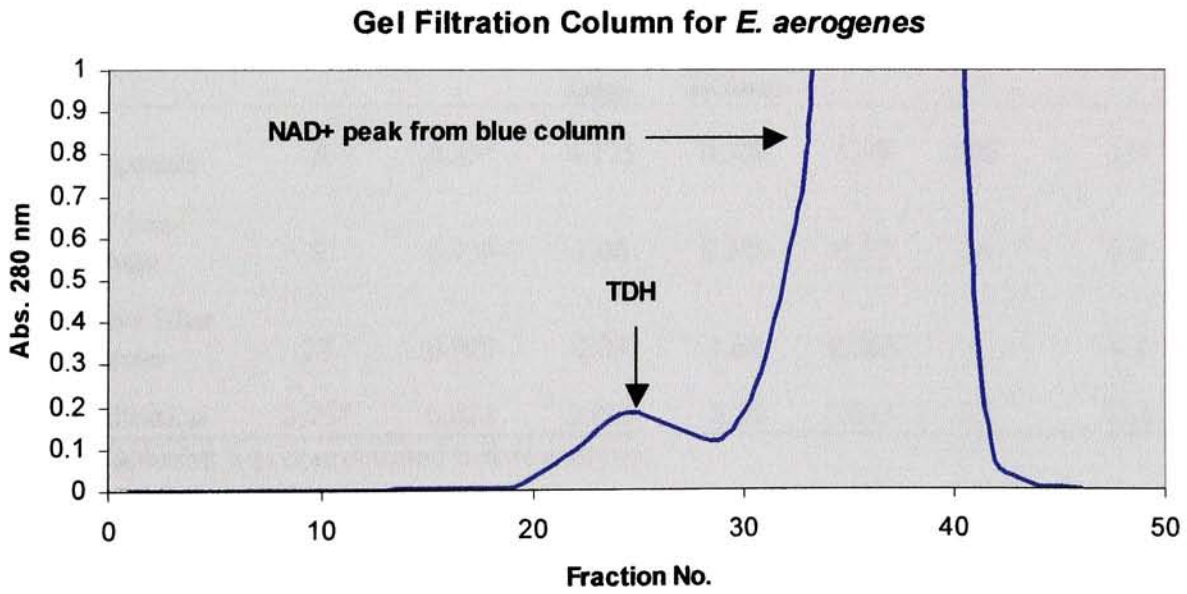


Figure 4.4 Elution profile for TDH activity from gel filtration column. Injection volume: 2 mL, Flow rate: 1 mL/min, Buffer: 20 mM Tris pH 8.5 with 100 mM KCl. Detector wavelength at 280 nm, AUFS 1.0. Fraction collector: 1 mL/tube. TDH activity was detected at fractions 23-27

Table 4.1
Summary of TDH concentration and activity assays for *K. pneumoniae*

Fraction	Volume (mL)	Protein (mg/mL)	Total protein (mg)	Specific Activity (U/mg)	Total Units	Yield (%)	Purification (fold)
Crude Homogenate	5	0.698	3.49	0.409	1.43	100	1
DEAE Ion-Exchange	8	0.114	0.912	1.11	1.01	71	2.7
Reactive Blue Sepharose	3*	0.040	0.12	2.10	0.25	17	5.1
Gel Filtration	0.75*	0.0067	0.005	24.1	0.12	8	59

* The solution was concentrated before analysis.

Table 4.2
Summary of TDH concentration and activity assays for *E. aerogenes*

Fraction	Volume (mL)	Protein (mg/mL)	Total protein (mg)	Specific Activity (U/mg)	Total Units	Yield (%)	Purification (fold)
Crude Homogenate	5	0.835	4.175	0.380	1.59	100	1.0
DEAE Ion-Exchange	8	0.133	1.06	0.549	0.58	36	1.4
Reactive Blue Sepharose	2*	0.020	0.04	1.58	0.063	4	4.2
Gel Filtration	0.75*	0.011	0.008	5.18	0.043	2.7	13.6

* The solution was concentrated before analysis.

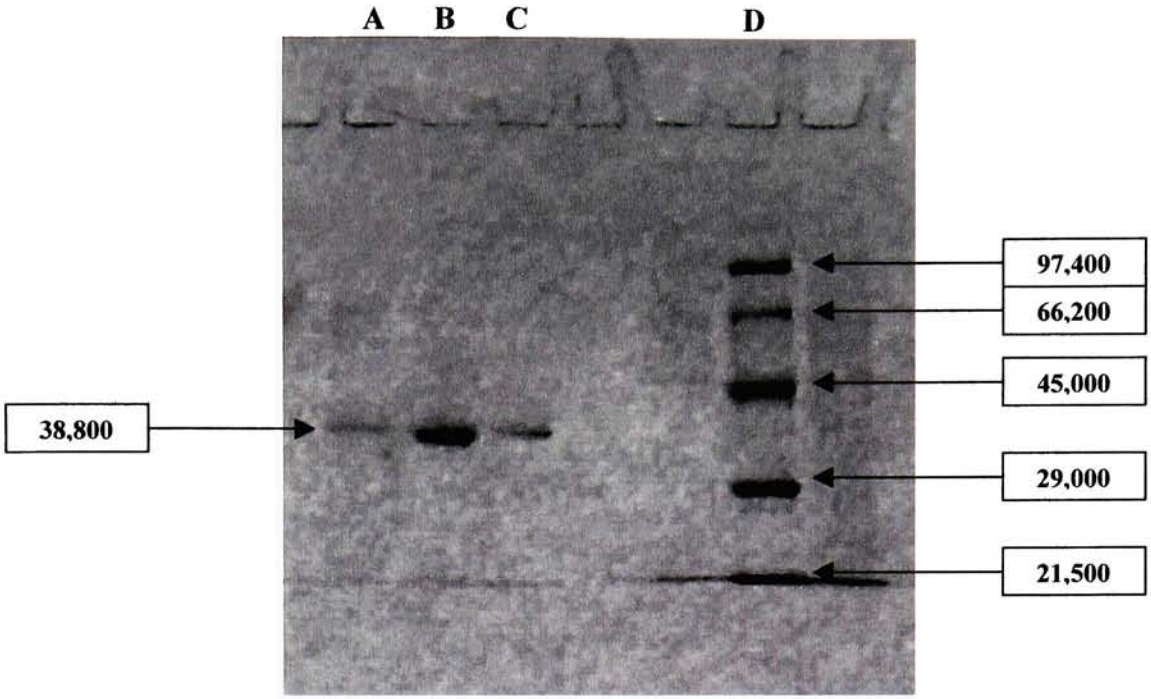


Figure 4.5 SDS-PAGE for *K. pneumoniae* TDH

Lane A: Pure TDH (0.1 mg/mL), Lane B: Pure TDH (0.3 mg/mL), Lane C: Pure TDH (0.2 mg/mL), Lane D: MW Standards.

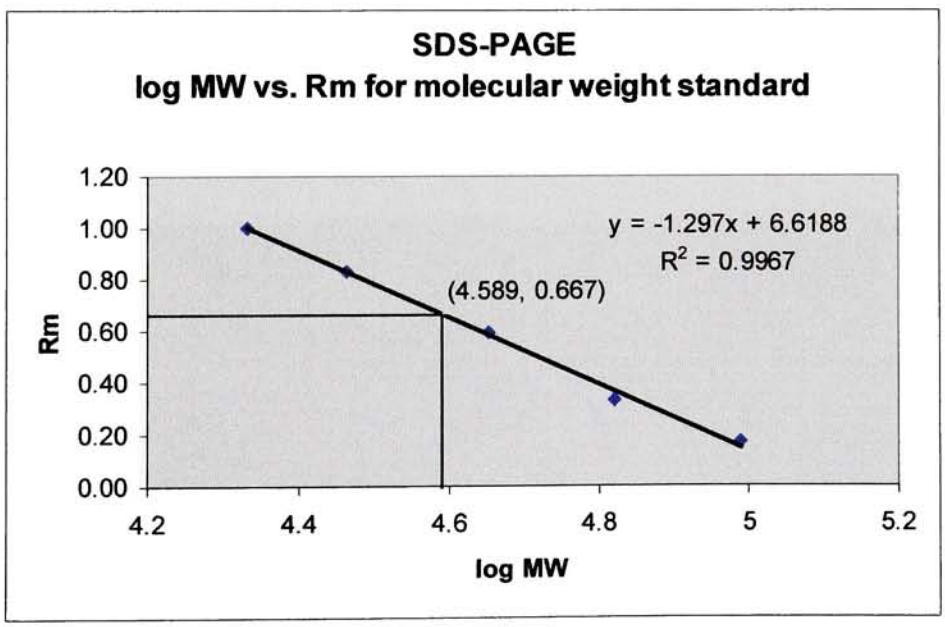


Figure 4.6 SDS-PAGE Calibration Curve

Log MW VS. R_m for Molecular Weight Standard

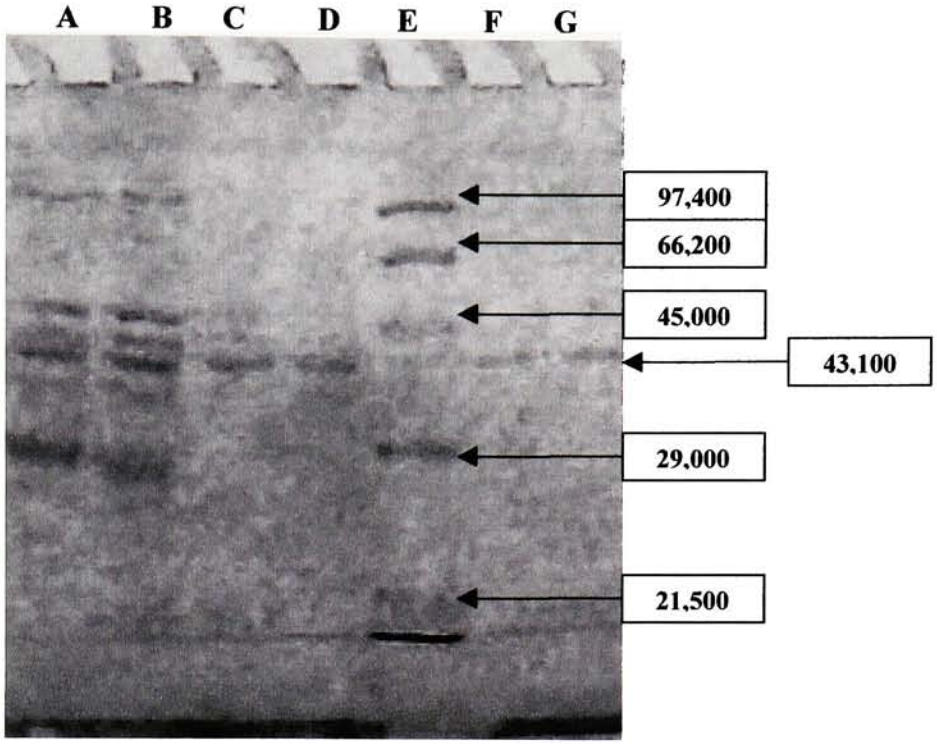


Figure 4.7 SDS-PAGE for *E. aerogenes* protein
Lane A and B: DEAE pool, Lane C and D: Blue affinity column pool, Lane E: MW Standards, and Lane F and G: pure TDH

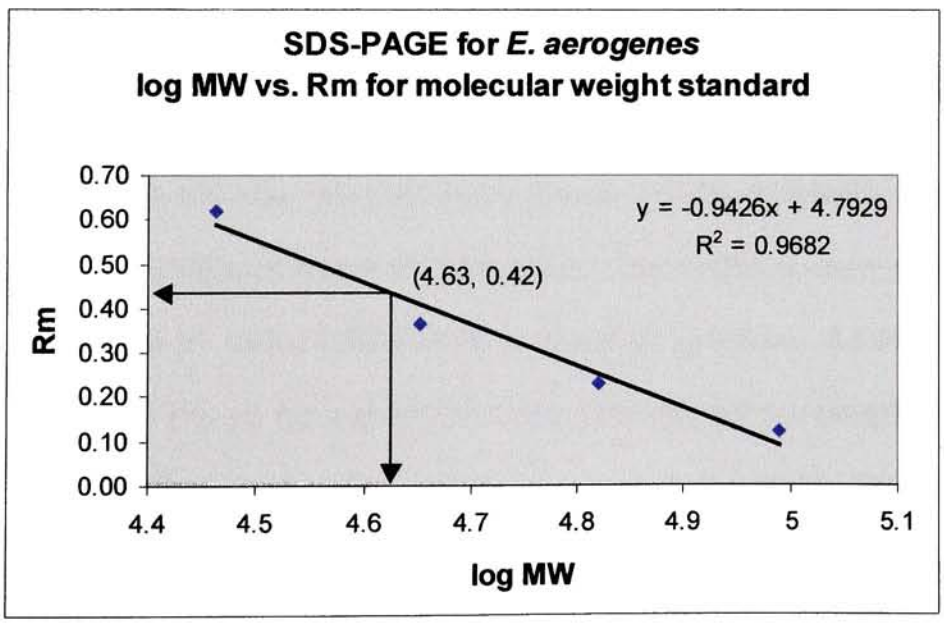


Figure 4.8 SDS-PAGE Calibration Curve
Log MW VS. R_m for Molecular Weight Standard

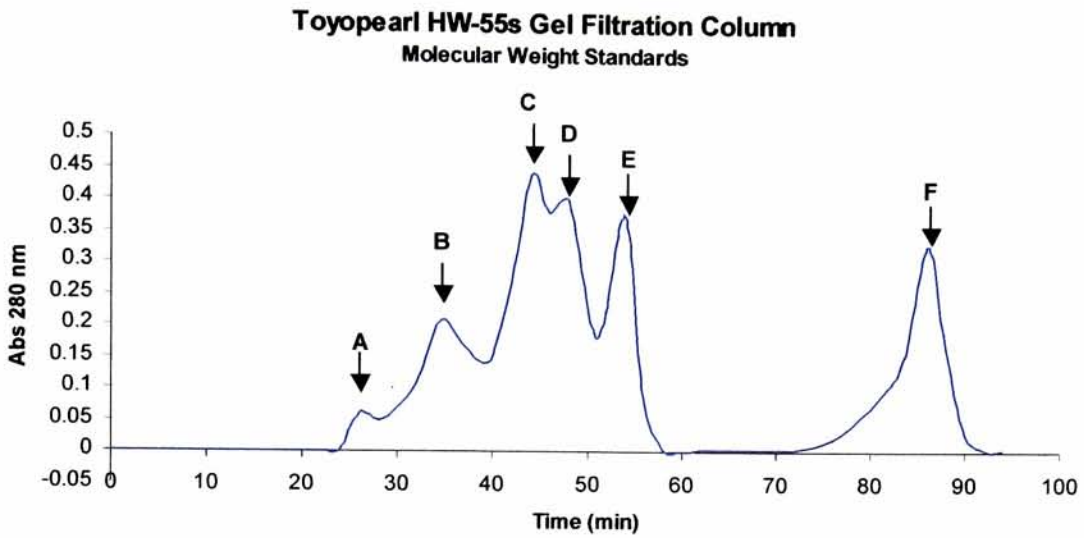


Figure 4.9 Molecular weight standards curve using gel filtration

	Standard	MW
A	Void Volume	-
B	Thyroglbulin	670,000
C	IgG	158,000
D	Ovalbumin	44,000
E	Myoglobin	17,000
F	Cyanocobalamin	1,350

contain thyroglbulin 670 kDa, IgG 158 kDa, ovalbumin 44 kDa, myoglobin 17 kDa and cyanocobalamin 1.35 kDa as seen in the table above. The conditions under which those standards as well as the native TDH proteins were run are as follows: 0.5 mL/min flow rate, buffer 20 mM Tris pH 8.5 with 100 mM KCl. The detector wavelength was set at 280 nm, and AUFS 1.0. A plot of log MW vs. R_m was used to calculate the native MW of the protein. The native MW estimate for *K. pneumoniae*'s TDH is (154 kDa) which is

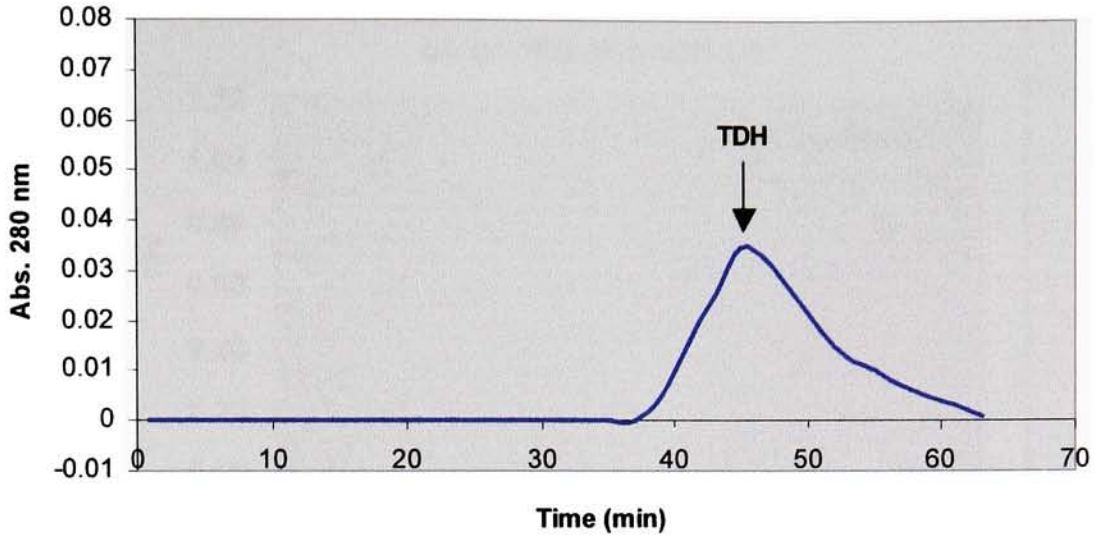


Figure 4.10 *K. pneumoniae* MW estimate

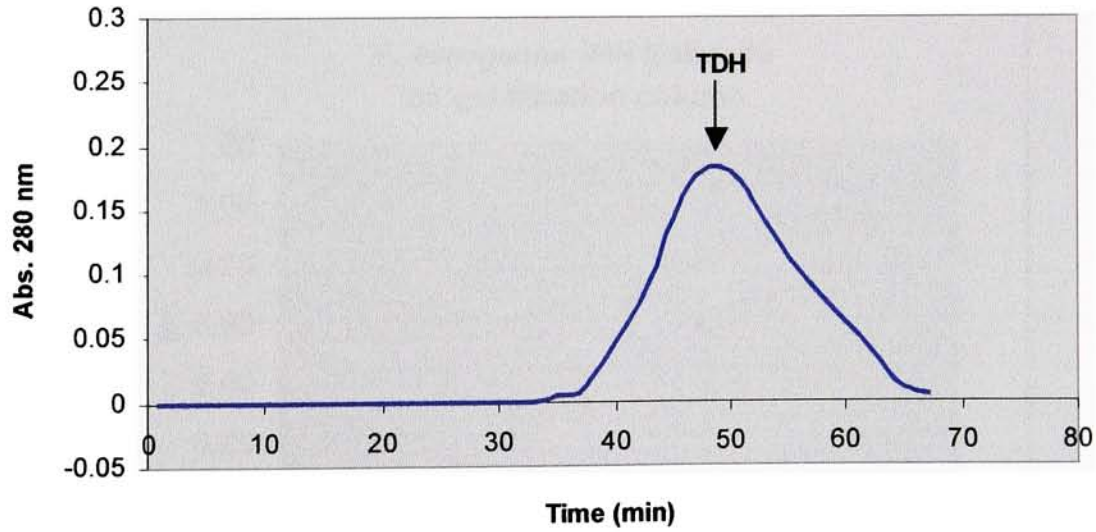


Figure 4.11 *E. aerogenes* MW estimate

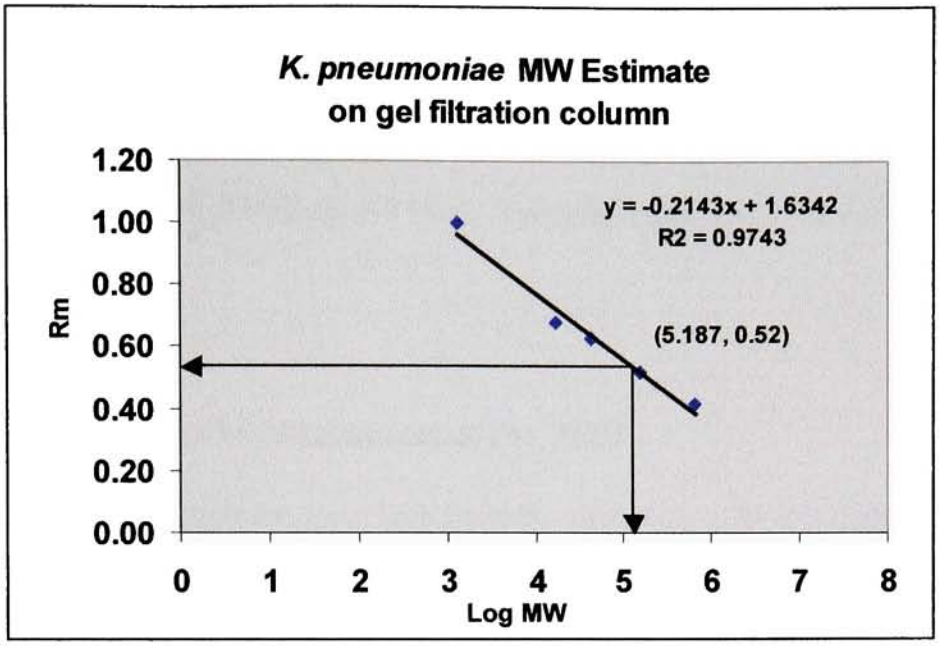


Figure 4.12 Gel filtration Calibration Curve for *K. pneumoniae*
Log MW VS. R_m for Molecular Weight Standard

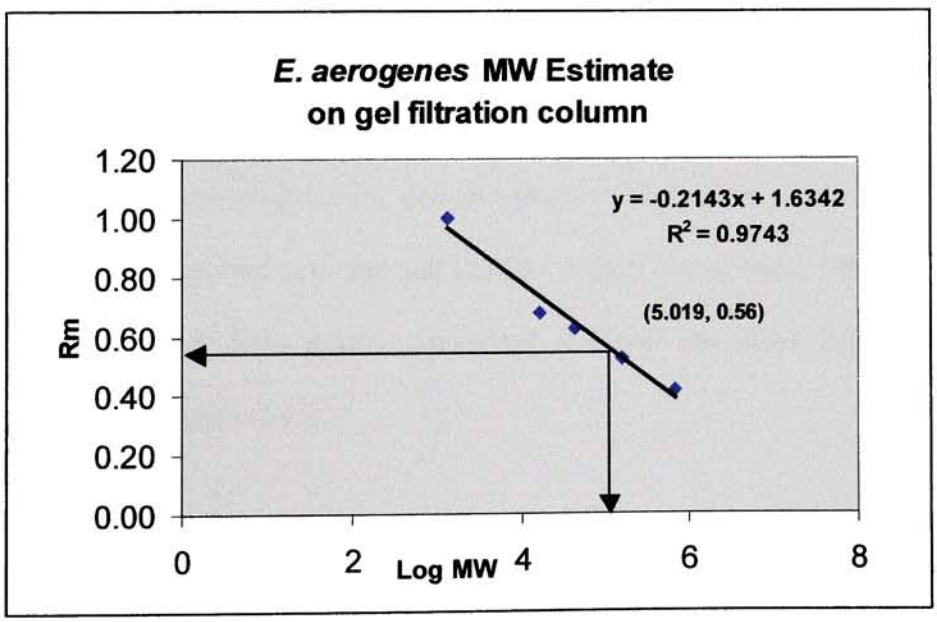


Figure 4.13 Gel filtration Calibration Curve for *E. aerogenes*
Log MW VS. R_m for Molecular Weight Standard

roughly four times the subunit size indicated from the SDS-PAGE estimate. This indicates that the native enzyme is a tetramer. As for *E. aerogenes*, the native protein's MW was calculated to be (104.5 kDa) which is roughly twice the size of the subunit estimate from the SDS-PAGE gel (43 kDa). This may indicate that the native enzyme is a dimer.

4.2 Sequencing the N-terminus of the TDH

The purified proteins, from both bacteria, were sent to Wyeth-Lederle Vaccines for an N-terminal sequence. The final sequencing results were 28 amino acids (MKALSKLKAE EGIWMTDVPE PEVGHNDL) for *K. pneumoniae* and 27 amino acids (MKAAVVTHD HQIDVTDKK LRRLEHG.A) for *E. aerogenes*. A BLAST search of both sequences in the National Center for Biotechnology Information[43,18] (NCBI) web site yielded several closely matching sequences that were aligned as seen in figures (4.14 and 4.15)

Figure 4.14 is the alignment of the N-terminal sequence from *K. pneumoniae* protein with other bacterial threonine dehydrogenases. Out of the 28 amino acids, there were five strictly conserved residues and the five highly conserved residues. As for *E. aerogenes*, there were four strictly conserved residues and eight highly conserved residues, as seen in (figure 4.15).

	1	28
Klebsiella pneumoniae	MKALSKLK AEEGIWMTDV PEPEVGHNDL	
Echerichia coli	MKALSKLK AEEGIWMTDV PVPELGHNDL	
Streptomyces coelicolor	MKALVKEN AEPGLWLADV PEPTIGSGDV	
Rhizobium meliloti	MKALVKTK PEVGLWMERV PVPEIGPNDV	
Deinococcus radiodurans	MRALSKQQ PGEGIWMIET EVPTPGPNDL	
Xanthomonas campestris	MKALVKRE ASKGIWLEQV PVPTPGPNEV	
Bacillus subtilis	MKALMKKD GAFGAVLTEV PIPEIDKHEV	
Pyrococcus abyssi	MVAIMKTK PAYGAELVEV DVPKPGPGEV	

Figure 4.14

Alignment[21] of *K. pneumoniae* N-terminal sequence with other bacterial threonine dehydrogenases. The 5 strictly conserved residues and the 5 highly conserved residues are indicated with blue and red colors respectively.

Enterobacter aerogenes	MKAAVVTHD. .HQIDVTDKK LRRLEHG~A
***Echerichia coli* (ADH I)**	MKAAVVTKD. .HHVDVTYKT LRSLKHGEA
***Zymomonas mobilis* (ADH I)**	MKAAVITKD. .HTIEVKDTK LRPLKYGEA
Serratia marcescens	MKAAVVTKN. .HTVDIQDKV LR~~~~~~~
***Neisseria meningitides* (ADH I)**	MQAVVVNKNV AGDVEVVERE VRPLEYGEA

Figure 4.15

Alignment[21] of *E. aerogenes* N-terminal sequence with similar bacterial sequences. The 4 strictly conserved residues and the 8 highly conserved residues are indicated with blue and red colors respectively. The dashed lines indicate absence of amino acids due to unknown sequences. The gaps represented by (.) were introduced to optimize alignment between sequences.

4.3 Sequencing the TDH gene using PCR

The second approach that was taken to sequence the TDH gene was to use *E. coli* primers 031, 030, 636, and 103 (figure 4.13) to amplify the gene from the *K. pneumoniae* genome. Primes 031 and 030 are located just outside the open reading frame of the *E. coli* TDH gene, whereas primers 636 and 103 were designed to amplify partial segments of the TDH gene. Figure 4.14 shows the amplified gene segment from *K. pneumoniae* using primers (031, 030) at two different annealing temperatures 37 °C and 40 °C. Figure 4.15 shows the PCR amplification results of partial segment of the TDH gene using (636, 103) and (636, 030) primers. The gene segment between primers (636, 103) is roughly 700 bp, and the segment between (636, 030) is roughly 900 bp. The attempts to amplify the gene segment between (031, 103) were unsuccessful.

The amplified gene segments were sent to the University of Rochester Core Nucleic Acid Laboratory for sequencing. Figure 4.16, 4.17, and 4.18 are the BLAST search results, which clearly indicate the similarities and differences between the query sequence and the subject sequence. After carefully examining the sequences, the ambiguous nucleotides represented by (n), the gaps (-) and the mismatches between the nucleotides were easily confirmed by comparing them with the results of the other primers.

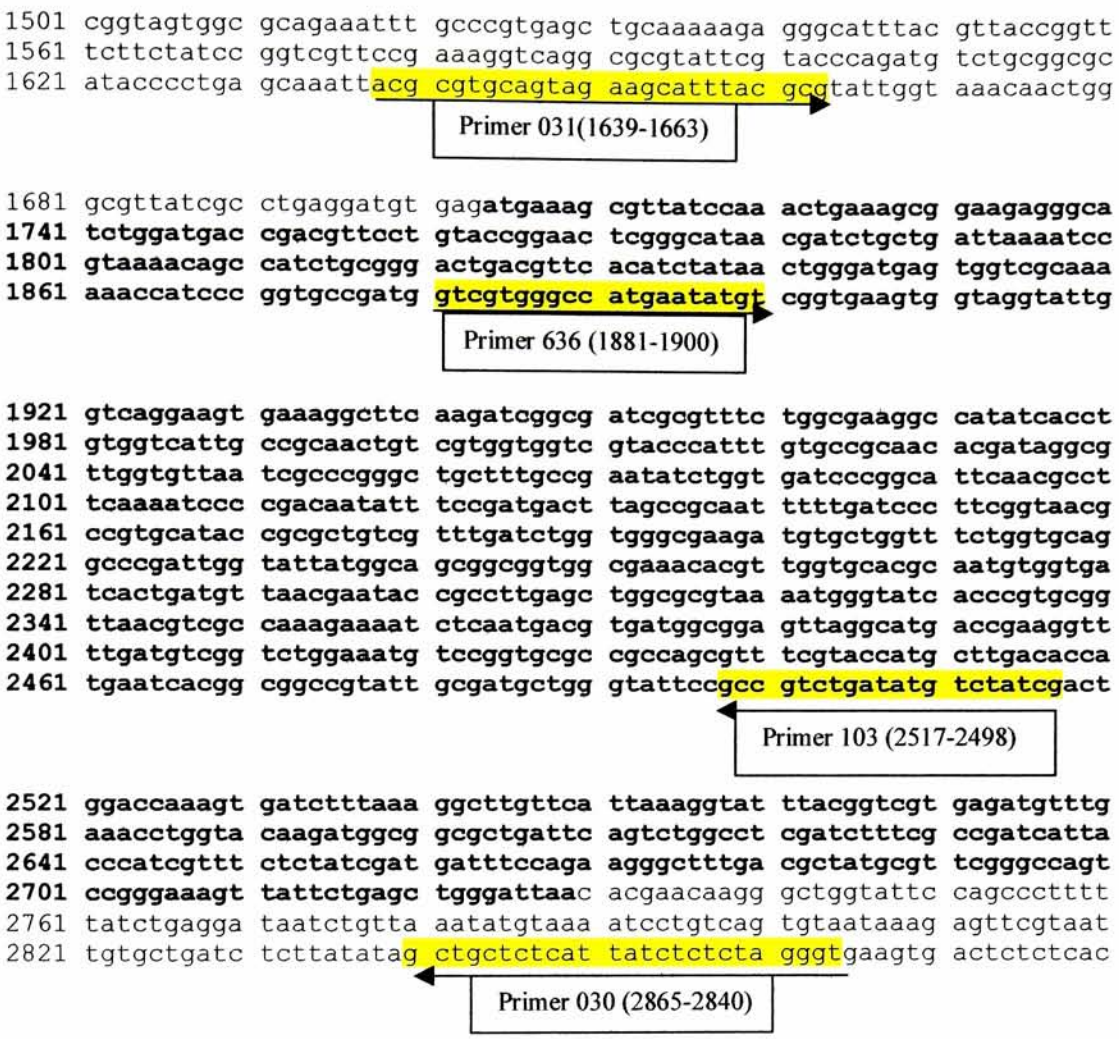


Figure 4.16
TDH gene of *E.coli*

Boldface letters indicate the complete sequence of the TDH gene from *E.coli* (TDH gene starts from 1704-2760). The arrows indicate the positions of the four sequencing primers and their sequencing direction.

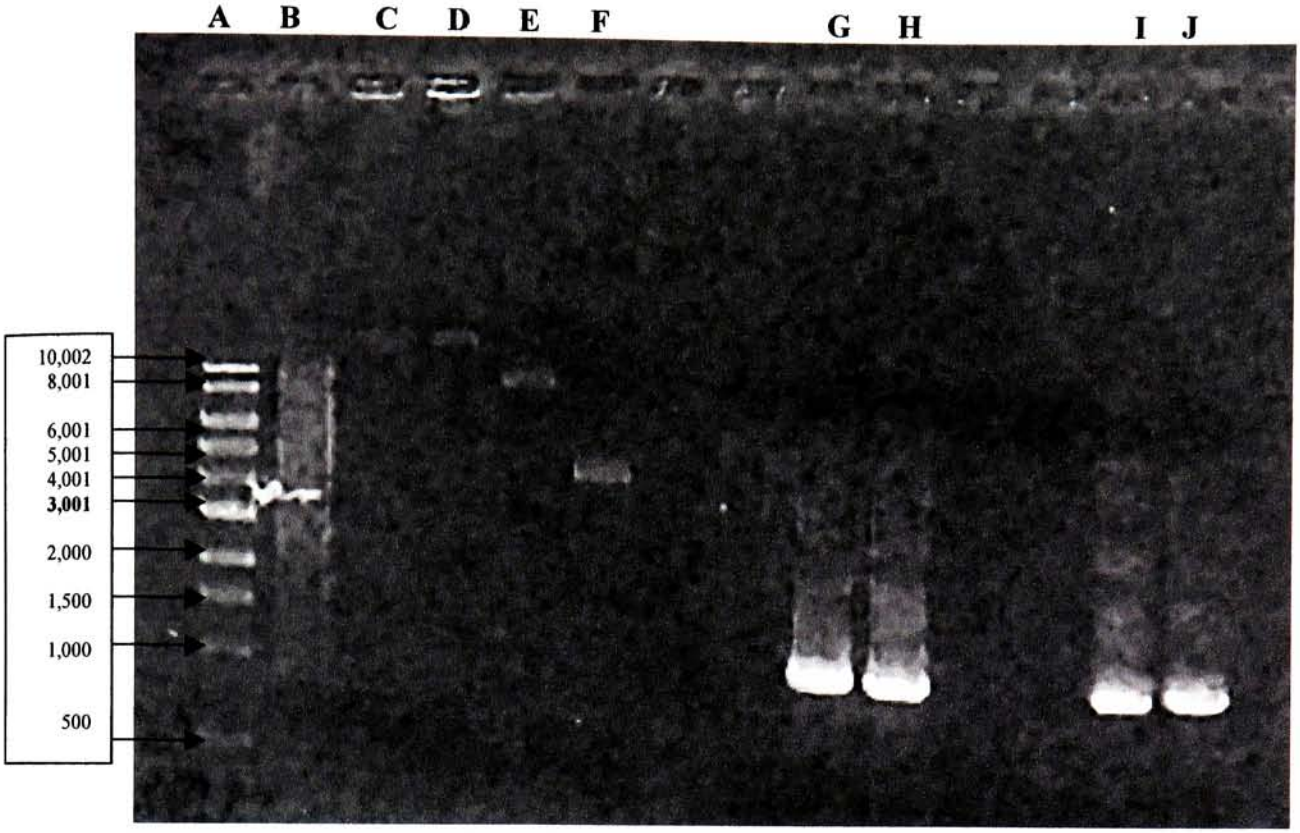


Figure 4.17
Agarose gel electrophoresis for TDH gene amplification from *k. pneumoniae* genomic DNA using primers (031, 030)

Lane	Name	Primers used	Size (bp)
A	DNA Ladder	-	(500-10,002)
B	λDNA/HindIII	-	-
C and D	*K. pneumoniae* genomic DNA	-	<10,000
E	SBD76-pDR121 Plasmid	-	≈10,000
F	SP1192-pAJWT plasmid	-	≈ 4,000-5,000
G	Control (37 °C annealing temp.)	031, 030	≈ 1,000-1,200
H	TDH gene (37 °C annealing temp.)	031, 030	≈ 1,000-1,200
I	Control (40 °C annealing temp.)	031, 030	≈ 1,000-1,200
J	TDH gene (40 °C annealing temp.)	031, 030	≈ 1,000-1,200

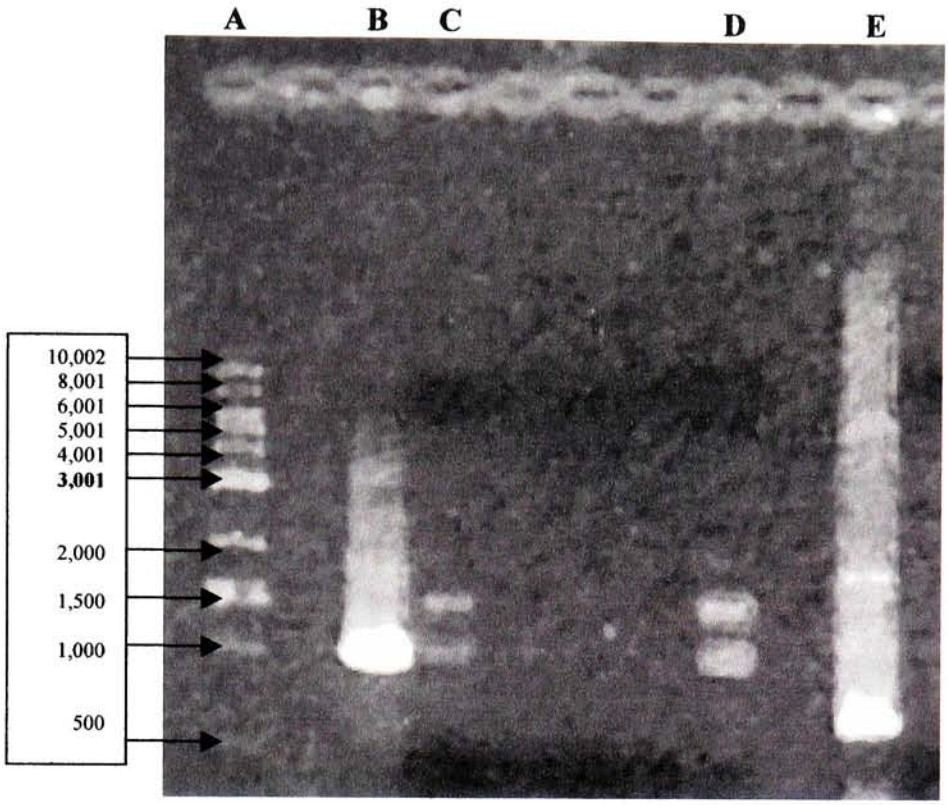


Figure 4.18
Agarose gel electrophoresis for TDH gene amplification from *k. pneumoniae* genomic DNA using primers (636, 030) and (636, 103)

Lane	Name	Primers used	Size (bp)
A	DNA Ladder	-	(500-10,002)
B	control	030, 636	≈ 900-1,000
C	Partial TDH gene using *Kp* genome as a template	030, 636	≈ 1,500 and 900-1,000
D	Partial TDH gene using *Kp* genome as a template	030, 636	≈ 1,500 and 900-1,000
E	Partial TDH gene using *Kp* genome as a template	103, 636	≈ 700

>emb|X06690.1|ECKBLTDH *E. coli* genes TDH and kbl
Length = 3563
Score = 1144 bits (577), Expect = 0.0
Identities = 598/603 (99%), Gaps = 2/603 (0%)
Strand = Plus / Plus

```
Query: 6    tggtaggtattggtcaggaagtgaaaggcttcaagatcggcgatcgcgtttctggcgaag 65
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1909 tggtaggtattggtcaggaagtgaaaggcttcaagatcggcgatcgcgtttctggcgaag 1968


Query: 66   gccatatcacctgtggtcattgccgcaactgtcgtggtggtcgtacccatttgtgccgca 125
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1969 gccatatcacctgtggtcattgccgcaactgtcgtggtggtcgtacccatttgtgccgca 2028


Query: 126  acacgataggcgttggtgttaatcgcccgggctgctttgccgaatatctggtgatcccgg 185
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2029 acacgataggcgttggtgttaatcgcccgggctgctttgccgaatatctggtgatcccgg 2088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Sbjct: 2149 ccttcggtaacgccgtgcataccgcgctgtcgtttgatctggtgggcgaagatgtgctgg 2208


Query: 306  tttctggtgcaggcccgattggtattatggcagcggcggtggcgaaacacgttggtgcac 365
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2209 tttctggtgcaggcccgattggtattatggcagcggcggtggcgaaacacgttggtgcac 2268


Query: 366  gcaatgtggtgatcactgatgttaacgaataccgccttgagctggcgcgtaaaatgggta 425
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2269 gcaatgtggtgatcactgatgttaacgaataccgccttgagctggcgcgtaaaatgggta 2328


Query: 426  tcacccgtgcggttaacgtcgccaaagaaaatctcaatgacgtgatggcggagttaggca 485
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2329 tcacccgtgcggttaacgtcgccaaagaaaatctcaatgacgtgatggcggagttaggca 2388


Query: 486  tgaccgaaggttttgatgtcggtctggaaatgtccngtgcgccgccagcgtttcgtacca 545
            |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct: 2389 tgaccgaaggttttgatgtcggtctggaaatgtccggtgcgccgccagcgtttcgtacca 2448


Query: 546  tgctttgacaccatgaatcacggcggccgtattgcgat-cttggtattncgccgtctgat 604
            ||| |||||||||||||||||||||||||||||||| || |||||| ||||||||||||
Sbjct: 2449 tgc-ttgacaccatgaatcacggcggccgtattgcgatgctgggtattccgccgtctgat 2507


Query: 605  atg 607
            |||
Sbjct: 2508 atg 2510
```

Figure 4.19
Sequence data from TDH 1909-2510 using primer 636

```
>emb|X06690.1|ECKBLTDH E. coli genes tdh and kbl
Length = 3563
Score = 1126 bits (568), Expect = 0.0
Identities = 589/596 (98%), Gaps = 1/596 (0%)

Query: 2    tcgcaatacggccgccgtgattcatggtgtcaagcatggtacgaaacgctggcggcgcac 61
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2484 tcgcaatacggccgccgtgattcatggtgtcaagcatggtacgaaacgctggcggcgcac 2425


Query: 62   cggacatttccagaccgacatcaaaaccttcggtcatgcctaactccgccatcacgtcat 121
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2424 cggacatttccagaccgacatcaaaaccttcggtcatgcctaactccgccatcacgtcat 2365


Query: 122  tgagattttctttggcgacgttaaccgcacgggtgatacccattttacgcgccagctcaa 181
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2364 tgagattttctttggcgacgttaaccgcacgggtgatacccattttacgcgccagctcaa 2305


Query: 182  ggcggtattcgttaacatcagtgatcaccacattgcgtgcaccaacgtgtttcgccaccg 241
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2304 ggcggtattcgttaacatcagtgatcaccacattgcgtgcaccaacgtgtttcgccaccg 2245


Query: 242  ccgctgccataataccaatcgggcctgcaccagaaaccagcacatcttcgcccaccagat 301
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2244 ccgctgccataataccaatcgggcctgcaccagaaaccagcacatcttcgcccaccagat 2185


Query: 302  caaacgacagcgcggtatgcacggcgttaccgaagggatcaaaaattgcggctaagtcat 361
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2184 caaacgacagcgcggtatgcacggcgttaccgaagggatcaaaaattgcggctaagtcat 2125


Query: 362  cggaaatattgtcggggattttgaaggcgttgaatgccgggatcaccagatattcggcaa 421
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2124 cggaaatattgtcggggattttgaaggcgttgaatgccgggatcaccagatattcggcaa 2065


Query: 422  agcagcccgggcgattaacaccaacgcctatcgtgttgcggcacaaatgggtacgaccac 481
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2064 agcagcccgggcgattaacaccaacgcctatcgtgttgcggcacaaatgggtacgaccac 2005


Query: 482  cacgacagttgcggcaatgaccacangtgatatggccttcgccagaaacgcgatcgncga 541
             |||||||||||||||||||||| |||||||||||||||||||||||||||||| |||
Sbjct: 2004 cacgacagttgcggcaatgaccacaggtgatatggccttcgccagaaacgcgatcgccga 1945


Query: 542  tctttaanccttca-tttctgaccaatacctaccacttnaccgacatattcatgg 596
             |||| || ||||||| || |||||||||||||||||| ||||||||||||||||
Sbjct: 1944 tcttgaagccttcacttcctgaccaatacctaccacttcaccgacatattcatgg 1889
```

Figure 4.20
Sequence data from TDH 1889-2484 using primer 103

```
>emb|X06690.1|ECKBLTDH E. coli genes tdh and kbl
Length = 3563
Score =  349 bits (176), Expect = 1e-93
Identities = 236/255 (92%), Gaps = 4/255 (1%)

Query: 6    tggtaggtattggtcaggaagtgaaaggcttcaagatcggcgatcgcgtttctggctgcg 65
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||   |
Sbjct: 1909 tggtaggtattggtcaggaagtgaaaggcttcaagatcggcgatcgcgtttctggcgaag 1968


Query: 66   gccatatcacctgtggtcattgccgcaactgtcgtggaaggtcgaacccatttgtgccgn 125
             |||||||||||||||||||||||||||||||||  ||||| |||||||||||||
Sbjct: 1969 gccatatcacctgtggtcattgccgcaactgtcgtggt-ggtcgtacccatttgtgccgc 2027


Query: 126  aacacgataggcgttggtgtnaatcgnccgggctgctttgccgaatatctggtgatcccg 185
             |||||||||||||||||| ||||| ||||||||||||||||||||||||||||||
Sbjct: 2028 aacacgataggcgttggtgttaatcgccgggctgctttgccgaatatctggtgatcccg 2087


Query: 186  gcattcaacgccttcaaaatccncnacaataatttccgatgacttaancccgnaatttat 245
             |||||||||||||||||||| | ||||| ||||||||||||||   ||| ||||| |
Sbjct: 2088 gcattcaacgccttcaaaatcccccgacaat-atttccgatgactta--gccgcaattttt 2144


Query: 246  gatncctncggtaac 260
             ||| ||| |||||||
Sbjct: 2145 gatcccttcggtaac 2159
```

Figure 4.21
Sequence data from TDH 1909-2159 using primer 636

In summary, the three sequences showed a 100% match to *E. coli* TDH gene, which may very well indicate a DNA contamination in the either one of the following steps:

1) Bacterial stock and growth
2) Genomic DNA isolation
3) PCR amplification step

The bacterial stock and growth were investigated by growing the bacteria in each step of the procedure on an EMB agar media, which is designed to differentiate *E. coli*

cells from *K. pneumoniae* cells. Both bacteria can grow on EMB media however, *E. coli* forms a metallic green color (figure 4.19) while *K. pneumoniae* forms a purple color colonies (figure 4.20).

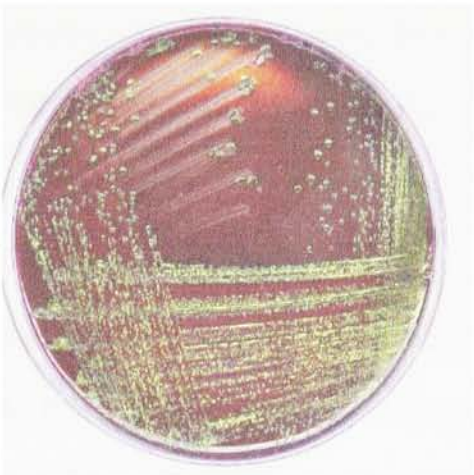

Figure 4.22
Growth of *E. coli* on EMB media

Figure 4.23
Growth of *K. pneumoniae* on EMB media

As for contamination during the isolation of *k. pneumoniae* genomic DNA, and during PCR amplification, a PCR reaction was preformed without any template DNA. If there was any trace of contamination with *E. coli* DNA, this reaction would have generated positive outcome. However, an agarose gel of the PCR reaction showed no amplified pieces of DNA. At this point of the research, the attempts to use PCR with *E. coli* primers to amplify the TDH gene from *k. pneumoniae* were unsuccessful.

4.4 Southern hybridization

Another approach was taken to try to sequence the gene from *K. pneumoniae* was southern hybridization. The first goal was to generate a gene probe that can be labeled with biotin and then use it to locate the TDH gene from a restriction digest reaction. Figure 4.21 shows the minimal gene, plasmid, and genomic DNA that can be detected with 100 ng probe/mL of hybridization solution (7mL/100cm^2). Table 4.3 indicates the amounts of DNA on the membrane for each spot and the positive or negative identity.

At this stage of this study, there were no further investigative attempts were made to obtain a DNA sequence of the TDH gene from *K. pneumoniae*.

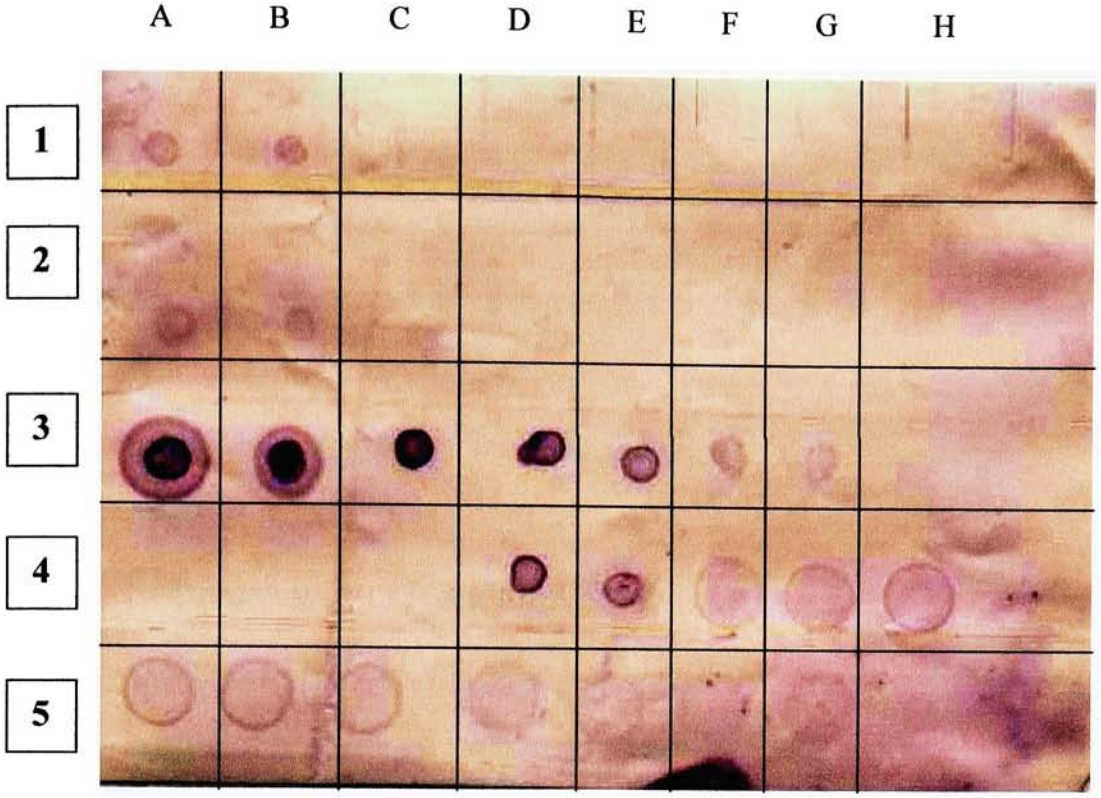

Figure 4.24
Nitrocellulose membrane showing hybridized spots of 1: pDR121 plasmid DNA, 2: pAJ plasmid DNA, 3: *E. coli* TDH gene, 4: (+) and (-) controls, 5: *K. pneumoniae* genomic DNA

Table 4.3

	DNA Type	A Lane	B Lane	C Lane	D Lane	E Lane	F Lane	G Lane	H Lane
1	pDR121 plasmid	(+) 140µg	(+) 100µg	(-) 50 µg	(-) 25 µg	(-) 12 µg	(-) 6 µg	(-) 3 µg	(-) 1.5 µg
2	pAJWT plasmid	(+) 100µg	(+) 50 µg	(-) 25 µg	(-) 12 µg	(-) 6 µg	(-) 3 µg	(-) 1.5 µg	
3	*E. coli* TDH gene	(+) 160µg	(+) 80 µg	(+) 40 µg	(+) 20 µg	(+) 10 µg	(+) 5 µg	(+) 2.5 µg	(-) 1.25µg
4	Control DNA	(-) λDNA	(-) λDNA	(-) λDNA	(+) Probe	(+) Probe	(+) Probe	(+) Probe	(+) Probe
5	*K p.* genomic DNA	(+) 400µg	(+) 360µg	(+) 320µg	(+) 280µg	(+) 240µg	(+) 200µg	(+) 160µg	(-) 120µg

CHAPTER V
DISCUSSION

In the early 1980s, an initial investigation on a variety of bacterial strains to test for threonine dehydrogenase activity showed a high level of activity in a *Klebsiella pneumoniae* cell crude extract based on a the aminoacetone assay.[44] Since then, there has been no further study on this topic. Therefore, the main goal was to sequence the TDH gene and characterize the enzyme from this organism.

5.1 Sequencing the TDH gene from *K. pneumoniae*

Our laboratory is heavily involved in the study of threonine dehydrogenase. Primers (030 and 031) were available to amplify and sequence the *E. coli* TDH gene, which have been used successfully before.[45] Having that in mind, the first option to sequence the same gene from *K. pneumoniae* was to use polymerase chain reaction with those primers to achieve this goal. Since those primers were designed for *E. coli* not for *K. pneumoniae*, the success rate for such experiment could not be predicted. That brought the first challenge, which was to try to find the best reaction conditions for PCR. Low annealing temperature (37-40) ^{o}C and different magnesium concentrations were tested to find the most suitable conditions. A positive outcome would be represented by a band size (1.0-1.2 kb) on an agarose gel. This band was sequenced and revealed a 100% identical to *E. coli*'s TDH gene. From that, it was concluded that the *K. pneumoniae* strain was contaminated with *E. coli* and therefore a new *Klebsiella pneumoniae subsp. Rhinoscleromatis* was purchased from the American Type Culture Collection®, ATCC # 13884. The reaction was repeated with the new bacteria, this time using two more inside

primers, 636 and 103 (see figure 4.16). Several bands were generated using a combination of the outside primers (031 and 030), the inside primers (636 and 103), and a combination of the outside-inside primers (031 and 103) and (636 and 030). All primers but (031 and 103) have generated bands. Those bands were excised and sent for sequencing. The only usable sequencing data received was the gene region between primers 636 and 103. Using BLAST search, the gene again matched 100% to *E. coli*'s TDH in that section of the gene. It is highly unlikely that two different bacteria have 100% match in the nucleotide sequence for a specific gene.

To further explore this possibility a Gene Bank and a BLAST search (table 5.1) was assembled to show some similar dehydrogenase sequences between *E. coli* and *K. pneumoniae* both in terms of nucleotides and amino acids.

Table 5.1
Similar dehydrogenases between *E. coli* and *K. pneumoniae*

Gene name	Nucleotide identity (%)	Amino acid similarity (Conserved)	(highly cons.)
6-Phosphogluconate dehydrogenase	1248/1334 (93%)	465/468 (99.3%)	466/468 (99.6%)
Glyceraldehyde 3-phosphate dehydrogenase	846/924 (91%)	277/290 (95.5%)	288/290 (99.3%)
Glutamate dehydrogenase	268/305 (87%)	270/304 (88%)	288/304 (93%)

From the above table, the highest gene homology between these two organisms was 93%. Based on these data, we concluded that the TDH gene from *K. pneumoniae* could not be 100% identical to the *E. coli* gene.

A different approach was taken to sequence the gene, this time using nonradioactive-southern hybridization to locate the TDH gene in *K. pneumoniae*. Among

all types of probes, gene probe provide the highest sensitivity. Therefore, a gene probe was generated using *E. coli*'s TDH gene. Random-primed synthesis was the first method that was used to generate the probe. This method resulted in a high level of non-specific signal on the Southern blot, leading us to use PCR to generate the gene probe. After numerous attempts and some minor protocol and material changes, a biotinylated gene probe was successfully generated using PCR. The probe detected as low as 2.5 μg of *E. coli*'s TDH gene, 100 μg from plasmid pDR121 (≈ 10,000 bp) and 50 μg from plasmid pAJWT (≈ 5,000 bp). Both pDR121 and pAJWT plasmids contain the *E. coli* TDH gene. However, the best result was the positive detection of a possible TDH gene on *K. pneumoniae*'s genome (< 25,000 bp) at a concentration of 160 μg. Due to time constrain, there were no further attempts to sequence the gene from *K. pneumoniae* and our interest shifted to sequencing the amino-terminus of the native protein.

5.2 TDH Characterization and N-Terminal Sequence

DEAE ion exchange chromatography was the first technique used in the purification procedure. This method separates molecules based on their net charge. Charged functional groups are covalently bound to a solid matrix with an anion exchanger such as DEAE. At a higher pH (8.5), the enzyme is negatively charged and can bind to the DEAE. At a flow rate of 4 mL/min and a running buffer of (20 mM Tris pH 8.5) with KCl gradient, TDH was eluted from the column and the activity was detected in both organisms around the same salt concentration (1.3-1.4) M KCl.

Dye-Ligand Affinity chromatography involves unique interactions between biomolecules. Since TDH is an NAD^+ dependent dehydrogenase, the theory of this

affinity column is based on this characteristic of the enzyme. The structure of Cibacron Blue 3GA mimics the negatively charged and aromatic structure of NAD^+. The samples were applied at low pH, (7.0) to increase positive charge on the enzyme, which favors binding to the dye. After washing at pH (7.0) to remove non-specifically bound proteins, buffer pH is raised to (8.5) to weaken TDH binding to the dye; NAD^+ is added to the buffer to displace the TDH in a sharp peak. The presence of NAD^+ (see figures 4.3 and 4.4) with the eluted TDH from this column supported this theory.

Gel filtration chromatography was the final step, which separates molecules based on molecular size. Optimum column running condition was found to be at flow rate of (0.5-1.0) mL/min and 20 mM Tris buffer (pH 8.5), which resulted in a well defined peaks and removed the NAD^+ that was added in the previous step.

In addition of being a purification step, this method provided an interesting point about the molecular weight of the native enzyme. Using molecular weight standards, a molecular weight estimate for the native enzyme was calculated to be (154 kDa) for *K. pneumoniae*. This is four times the size of the single band on the SDS-PAGE gel (38.8 kDa). This leads to the conclusion that the native form of the protein is a tetramer with four identical subunits. The size of this enzyme from *K. pneumoniae* resembles that of *E. coli*[46], which is also a tetramer and composed of four identical subunits (37.2 kDa), and (148 kDa) for the MW of the native protein. As for *E. aerogenes*, the native protein's MW was estimated to be (104.5 kDa). This is roughly twice the size of the single band from the SDS-PAGE gel (43 kDa). This suggests that the native protein is a dimer with two identical subunits.

The N-terminal sequence of the protein from *K. pneumoniae* was obtained using the Edman degradation chemistry. The resulting sequence (MKALSKLKAE EGIWMTDVPE PEVGHNDL) was compared to that of similar sequences using a BLAST search and yielded the results in (table 5.2) below:

Table 5.2

Comparisons of *K. pneumoniae*'s N-terminus (top sequence in each pair) with other known TDH sequences. The letters in blue color represent conserved amino acids between the two matches and the letters in red represent highly conserved amino acids.

Organism	Sequence	% homology to *Kp* TDH	% Similarity to *Kp* TDH
Klebsiella pneumoniae	MKALSKLKAE EGIWMTDVPE PEVGHNDL	(100%)	(100%)
Echerichia coli	MKALSKLKAE EGIWMTDVPE PEVGHNDL MKALSKLKAE EGIWMTDVPV PELGHNDL	26/28 (92%)	27/28 (95%)
Streptomyces coelicolor	MKALSKLKAE EGIWMTDVPE PEVGHNDL MKALVKENAE PGLWLADVPE PTIGSGDV	16/28 (57%)	20/28 (71%)
Rhizobium meliloti	MKALSKLKAE EGIWMTDVPE PEVGHNDL MKALVKTKPE VGLWMERVPV PEIGPNDV	17/28 (60%)	20/28 (70%)
Deinococcus radiodurans	MKALSKLKAE EGIWMTDVPE PEVGHNDL MRALSKQQPG EGIWMIETEV PTPGPNDL	15/28 (53%)	18/28 (64%)
Xanthomonas campestris	MKALSKLKAE EGIWMTDVPE PEVGHNDL MKALVKREAS KGIWLEQVPV PTPGPNEV	14/28 (50%)	19/28 (68%)
Bacillus subtilis	MKALSKLKAE EGIWMTDVPE PEVGHNDL MKALMKKDGA FGAVLTEVPI PEIDKHEV	10/28 (36%)	15/28 (54%)
Pyrococcus abyssi	MKALSKLKAE EGIWMTDVPE PEVGHNDL MVAIMKTKPA YGAELVEVDV PKPGPGEV	7/28 (25%)	13/28 (46%)

All seven of those bacterial TDH sequences are in the long-chain alcohol dehydrogenase family that contains a catalytic and a structural zinc atom. In addition, all native proteins are tetramers with four identical subunits. This only confirms the previous results generated in this study. Most interesting of all is the 92% and 95%

homology of the conserved and highly conserved amino acids between *K. pneumoniae*'s and *E. coli*'s first 28 amino acids of the N-terminal.

As for *E. aerogenes*, the N-terminal sequence (MKAAVVTHD HQIDVTDKK LRRLEHG.A) was also compared to that of similar sequences using a BLAST search and yielded the results in table 5.3 below:

Table 5.3

Comparisons of *E. aerogenes* N-terminus (top sequence in each pair) with other protein sequences. The letters in blue color represent conserved amino acids between the two matches and the letters in red represent highly conserved amino acids. The dashed lines indicate absence of amino acids either due to unknown sequences or differences in peptide length. The gap represented by (.) is a missing amino acid in the original *E. aerogenes* sequence.

Organism	Sequence	% Identity to *Ea* TDH	% Similarity to *Ea* TDH
Enterobacter aerogenes	MKAAVVTHD HQIDVTDKK LRRLEHG.A	100%	100%
***Echerichia coli* (ADH I) PROPANOL-PREFERRING**	MKAAVVTHD HQIDVTDKK LRRLEHG.A MKAAVVTKD HHVDVTYKT LRSLKHGEA	21/29 (72%)	23/29 (79%)
***Zymomonas mobilis* (ADH I) PROPANOL-PREFERRING**	MKAAVVTHD HQIDVTDKK LRRLEHG.A MKAAVITKD HTIEVKDTK LRPLKYGEA	20/29 (69%)	23/29 (79%)
Serratia marcescens	MKAAVVTHD HQIDVTDKK LRRLEHG.A MKAAVVTKN HTVDIQDKV LR~~~~~~~	13/20 (65%)	16/20 (80%)
***Neisseria meningitidis* (ADH I) PROPANOL-PREFERRING**	MKAAVVTHD~ ~HQIDVTDKK LRRLEHG.A MQAVVVNKNV AGDVEVVERE VRPLEYGEA	10/29 (34%)	18/29 (62%)

Except for *Serratia marcescens*[47], the sequences in (table 5.3) for *Echerichia coli*[48], *Zymomonas mobilis*[49], and *Neisseria meningitides*[50] are known to belong to the (ADH I) propanol-preferring family. They are dimers that contain a catalytic and a structural zinc atom in each subunit.

Using the GCG programs, a Neighbor-Joining phylogenetic tree (figure 5.1) was constructed for *K. pneumoniae* and the other seven organisms to elucidate their evolutionary relationships based on the first 28 amino acids of the N-terminal sequence. It is important to note that 28 amino acids may not be sufficient data to build a conclusion on the validity of the tree. A similar tree (figure 5.2) was generated for *E. aerogenes* and the other propanol-preferring ADHs.

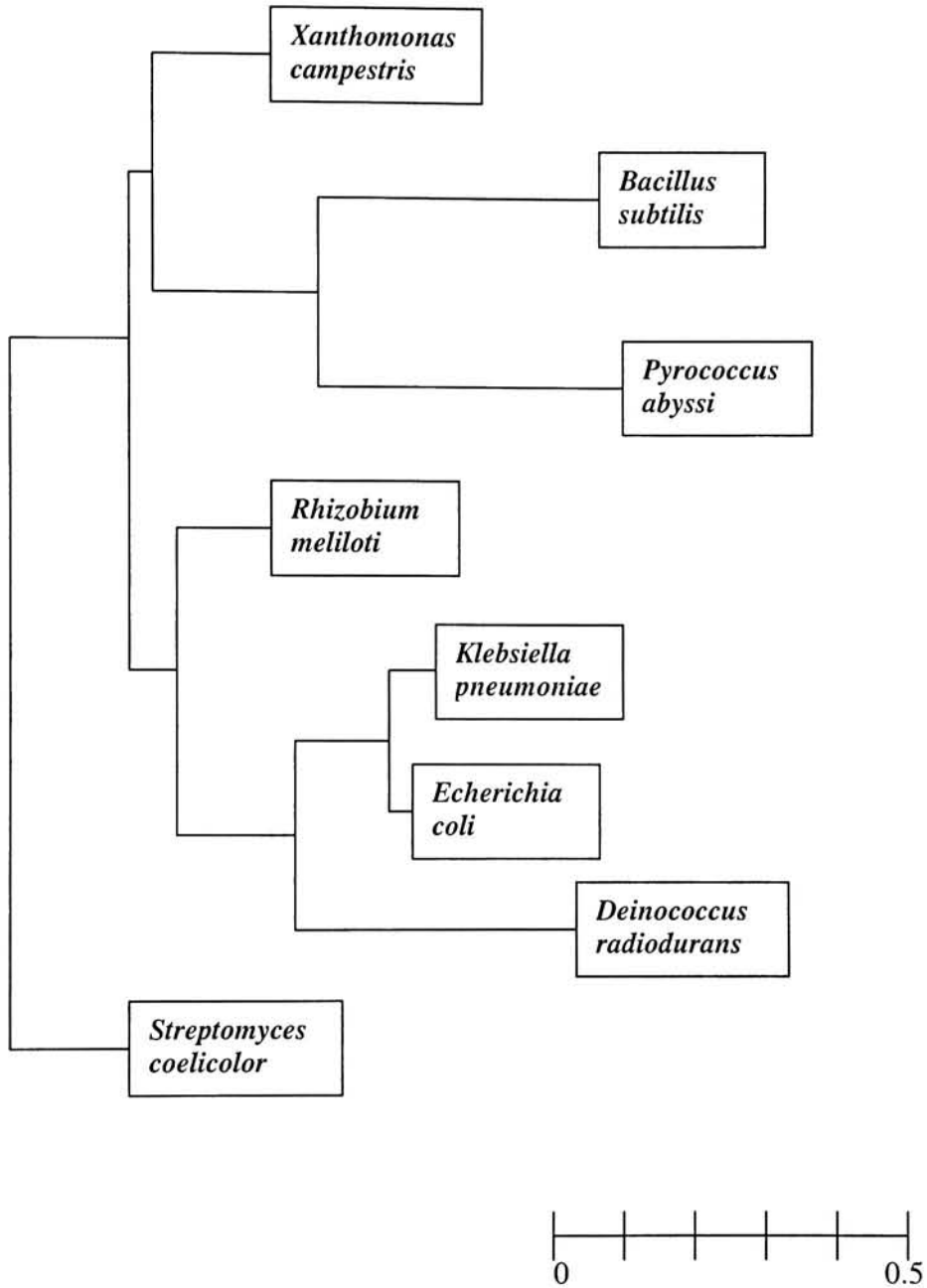

Figure 5.1 Neighbor-Joining tree for bacterial TDH based on the first 28 amino acids of the amino terminus. This tree was generated using the Wisconsin Package of the Genetic Computer Group (GCG).

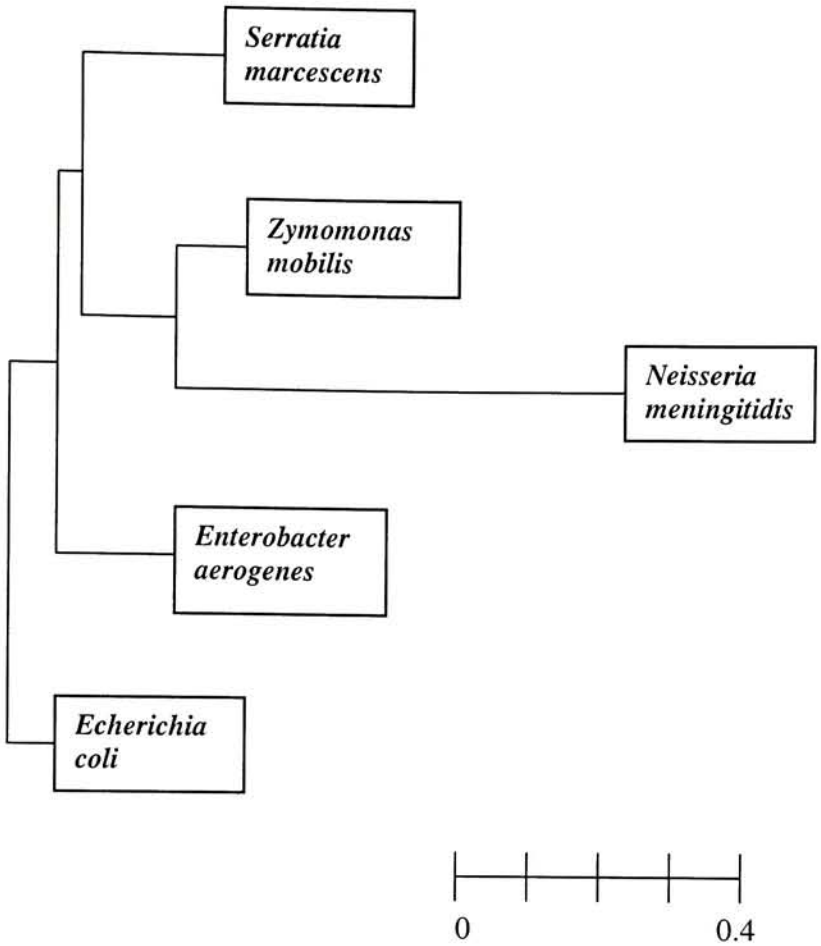

Figure 5.2 Neighbor-Joining tree for bacterial (ADH I) propanol-preferring, based on the first 29 amino acids of the amino terminus. This tree was generated using the Wisconsin Package of the Genetic Computer Group (GCG).

In conclusion, *K. pneumoniae*'s native TDH protein is a tetramer with a molecular weight estimate of 154 kDa. Like other known threonine dehydrogenases, TDH from *K. pneumoniae* has four subunits with a molecular weight of 38.8 kDa, which translates to approximately 352 amino acids per subunit. These results are consistent with organisms listed in (table 1.1 p 4). Since all TDHs from those organisms have two zinc atoms per enzyme subunit, it seems likely that *K. pneumoniae*'s TDH may also contain a catalytic

and a structural zinc in each subunit. The close relationship between *E. coli* and *K. pneumoniae* enzymes has also been shown in terms of the first 28 amino acid of the N-terminal.

Unlike the *K. pneumoniae* enzyme, TDH from *Enterobacter aerogenes* has a native molecular weight of 104.5 kDa with a subunit size of 43 kDa. Roughly, this translates to two subunits per enzyme with each subunit has approximately 390 amino acids in length. Although *E. aerogenes*'s TDH did not show a great deal of homology to *E. coli*'s TDH, it did have 34%-72% homology with other alcohol dehydrogenases; propanol-preferring (ADH I) in particular. Since threonine contains an isopropanol group, it is very likely that the protein from this organism is also a propanol-preferring, which may also contain two zinc atoms per subunit.

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